BIOACTIVE CROSSLINKED PROTEIN-BASED FILMS REINFORCED BY NANOPARTICLES

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A mi madre

INDEX

ABBREVIATIONS USED I

SUMMARY III

RIASSUNTO V

1. INTRODUCTION 1

1.1 Scientist context 3
1.2 Edible films 3
1.3 BV protein 5
1.4 Glycerol 6
1.5 Reinforced edible films 6
1.6 Nanotechnology 6
1.6.1 MSN 7
1.6.2 Biopolymers/Nanoclay film 8
1.6.3 mTGase-containing films 9
1.7 Film antimicrobial and antifungal additives 11
1.8 General objective of the experimental work 15

2. MATERIALS AND METHODS 16
2.1 Materials 17
2.2 NP characterization 17
2.3 FFS preparation and casting 18
2.3.1 Optimal amount of NPs 19
2.3.2 Effect of mTGase 19
2.3.3 Zeta-potential and particle size 19
2.3.4 Casting 19
2.4 Film morphological characterization 19
2.4.1 SEM 19
2.4.2 AFM 20
2.5 Film physicochemical characterization 20
2.5.1 Thickness measurements 20
2.5.2 DSC 20
2.5.3 TGA 21
2.5.4 FT-IR spectroscopy 21
2.5.5 Mechanical properties 21
2.5.6 Barrier properties 21
2.6 FFS and film biological properties 21
2.6.1 Antimicrobial activity 22
2.6.2 Antifungal activity 22
2.7 Statistical analysis 23

3. RESULTS & DISCUSSIONS 25
3.1 Preparation and characterization of NPs 26
3.1.1 MSN 26
3.1.2 APTES-MSN 27
3.2 Preparation and characterization of BVPC FFS and derived films 30
3.2.1 Characterization of FFS 30
3.2.2 Effect of the addition of different amount of NPs to BVPC FFS and derived films 31
3.2.2.1 Optimization of NP/BVPC ratio 31
3.2.2.2 Mechanical properties of NPs-BVPC films 31
3.3 Effect of mTGase-catalyzed protein crosslinking on the physicochemical properties of nano-reinforced BVPC films 33
3.3.1 Mechanical properties 34
3.3.2 Barrier properties 35
3.3.3 Morphological properties 36
3.3.4 Thermoanalytic properties 38
3.3.5 FT-IR characterization 41
3.3.6 Biological properties 42
3.3.6.1 Antimicrobial activity of BVPC FFSs 42
3.3.6.2 Antimicrobial activity of BVPC films 43
3.3.6.3 Antifungal activity of BVPC films 44

4. CONCLUSIONS 47

5. REFERENCES 51

6. APPENDIX 61

ABBREVIATIONS USED

(3-Aminopropyl) triethoxysilane	APTES
Atomic force microscopy	AFM
Bitter vetch protein concentrate	BVPC
Differential scanning calorimetry	DSC
Elongation at break	EB
Film forming solution	FFS
Fourier-transform infrared spectroscopy	FTIR
Mesoporous silica nanoparticle	MSN
Microbial transglutaminase	mTGase
Nanoparticle	NP
Polidispersity index	PDI
Scanning electron microscope	SEM
Tensile strength	TS
Thermogravimetric analysis	TGA
Transmission electron microscopy	TEM

SUMMARY

About 300 million tons of plastic wastes are produced annually in the world and, unfortunately, only 7% are recycled, the remaining 93% ending up in landfills and oceans. The environmental impact of plastic wastes is escalating rising widespread global concern since disposal systems are inadequate.
It is crucial to find enduring plastic alternatives, especially in short-term food packaging and disposable applications. One possible solution is the synthesis of bio-based and biodegradable/edible plastics. Nowadays, several research programs are trying to design new natural packaging materials for food products to contribute to the pollution reduction.
Moreover, edible films and coatings could carry preservatives with the aim of improving the surface microbial stability of foods. Microbial contamination may occur primarily at the food surface, due to post-processing handling, so the use of active packaging films containing antimicrobial agents could be a very efficient procedure, by slowing down migration of the agents from the packaging material to the surface of the product.
The present work was carried out by preparing edible films using bitter vetch (*Vicia ervilia*) protein concentrate (BVPC), an ancient grain legume crop of the Mediterranean region, as protein renewable source. In order to reinforce the mechanical properties of BVPC films and to improve their barrier features toward gases (CO_2 and O_2) and water vapor, mesoporous silica nanoparticles (MSNs) and their (3-aminopropyl) triethoxysilane (APTES) amino-derivatives were synthesized, characterized and integrated into the films. In addition, microbial transglutaminase (mTGase), a protein crosslinking enzyme, was added to the BVPC film forming solution (FFS) to reticulate the films by creating a more compact protein network structure. All the obtained films were physico-chemically and morphologically characterized and showed antimicrobial and antifungal activities. Finally, a more active biomaterial was obtained by incorporating into the reinforced films also nisin, a well known antimicrobial and antifungal agent.

RIASSUNTO

1. Introduzione

Circa 300 milioni di tonnellate di rifiuti di plastica vengono prodotti annualmente nel mondo e, purtroppo, solo il 7% viene riciclato mentre, il restante 93% finisce nelle discariche. L'impatto ambientale dei rifiuti plastici rappresenta un serio problema a livello globale e quindi, risulta sempre più di fondamentale importanza trovare alternative sostenibili alle plastiche di origine petrolifera da utilizzare in particolare nell'imballaggio alimentare. Una possibile soluzione a tale problema è rappresentato dall'utilizzo di materie plastiche biodegradabili (Falguera et al., 2011; Pathak et al., 2014). Infatti, la crescente preoccupazione per l'ambiente a livello mondiale, ha spinto il mondo della ricerca a sviluppare diversi programmi rivolti alla sintesi di nuovi materiali biodegradabili ottenuti da fonti rinnovabili, da proporre in alternativa a quelli di origine petrolifera. Tra questi materiali, rivestono particolare importanza i film edibili ottenuti da biopolimeri naturali come proteine, polisaccaridi e lipidi. Questi biopolimeri possono essere utilizzati da soli o in miscela, sia come film strutturati che come rivestimenti (*coatings)* formati direttamente sull'alimento. La principale funzione di questi film edibili è principalmente quella di proteggere l'alimento dall'ambiente circostante, potendo essere anche utilizzati come supporti o veicoli di molecole funzionali, quali antimicrobici e antiossidanti, e in questo caso essi vengono denominati "imballaggi attivi" (*active packaging*). La funzione degli imballaggi attivi ha lo scopo di migliorare la stabilità microbica della superficie degli alimenti e/o preservare questi ultimi dai fenomeni ossidativi (Pérez-Pérez, 2006).
Negli ultimi anni la nanotecnologia, grazie alle piccole dimensioni e le grandi aree di superficie esposte dalle nanoparticelle utilizzate, ha riscontrato un grande interesse nel settore alimentare e, in particolare, sia per nanoincapsulare composti bioattivi e fungere da loro *carrier* negli alimenti (Duran, 2013) sia nello sviluppo di materiali nano-rinforzati utili a migliorare le proprietà chimico-fisiche di diversi materiali, tra cui la resistenza meccanica, la stabilità termica e le proprietà di barriera (Arora et al., 2010).
In questo lavoro di tesi è stata studiata la possibilità di preparare film nanorinforzati con migliorate proprietà meccaniche e di barriera utilizzando come matrice il concentrato proteico estratto dai semi della veccia amara (*Vicia ervilia*) e come nanoparticelle la silica mesoporosa e un suo derivato amminato. I film sono stati poi ulteriormente rinforzati dalla formazione di legami covalenti tra le diverse molecole proteiche catalizzata dalla transglutaminasi microbica, un enzima in grado di produrre lagami covalenti tra residui endoproteici di lisina e glutammina. Infine è stata anche saggiata l'attività antimicrobica e antifungina dei film ottenuti nelle varie condizioni sperimentali in assenza e presenza della nisina, un oligopeptide dalle ben note attività biologiche.

2. Risultati

Sintesi delle Nanoparticelle di Silica Mesoporosa e loro funzionalizzazione

Le nanoparticelle di MSN sono state sintetizzate come riportato da Chen et al. (2011) utilizzando il bromuro di cetiltrimetilammonio come tensioattivo che permette di ottenere delle micelle che fungono da stampo per il gruppo silano (tetraetossisilano) che forma la struttura della nanoparticella. Una volta creata la nanoparticella, il tensioattivo è stato rimosso mediante calcinazione a 550 °C per 5 ore ottenendo così la silica mesoporosa. Analisi mediante microscopia a trasmissione e ai raggi X hanno evidenziato una distribuzione omogenea delle nanoparticelle del diametro di circa 100 nm che presentavano grandi pori uniformi evidenziati sia mediante miscroscopia che misurando le isoterme di assorbimento-desorbimento di azoto. Inoltre, la superficie delle nanoparticelle è stata funzionalizzata con gruppi amminici utilizzando il 3-amino-propil-trietossisilano (APTES) al fine di migliorare le interazioni tra particelle e matrice proteica (Yokoi et al., 2012). Le funzionalizzazione delle nanoparticelle è stata confermata mediante analisi FT-IR, calorimetria differenziale a scansione e misura del potenziale zeta. I risultati hanno messo in evidenza l'effettiva funzionalizzazione della superfice delle nanoparticelle.

Determinazione del pH ottimale per la solubilizzazione del concentrato proteico e di preparazione dei film

Per verificare le migliori condizioni di pH a cui era possibile ottenere una migliore solubilizzazione del concentrato proteico, è stata eseguita la misura del potenziale zeta della soluzione a diversi valori di pH (da pH 12 a pH 1). È ben noto che le proprietà dei materiali sono associate alla distribuzione omogenea dei composti nelle soluzioni filmanti e alla loro capacità di non aggregare prima dell'evaporazione del solvente. Questa proprietà può essere correlata ai risultati del potenziale zeta e può essere utile nella scelta del pH appropriato per realizzare i film (Sabbah et al., 2016). I risultati ottenuti hanno evidenziato che le migliori condizioni di solubilità della miscela proteica erano compresi tra pH 12 e pH 7. Al fine di valutare l'effetto del pH della soluzione proteica sulle proprietà dei film da essa derivati, sono stati preparati film mediante casting delle soluzioni ottenute a pH 7, 10 e 12. I risultati hanno evidenziato che i film con le proprietà meccaniche migliori erano ottenuti effettuando il casting a pH 12.

Proporzione ottimale di MSN e APTES-MSN per la preparazione dei film nanorinforzati

Al fine di studiare l'effetto di MSN e APTES-MSN sulla stabilità delle soluzioni e sulle proprietà meccaniche dei film da esse ottenuti, diverse quantità di MSN o APTES-MSN (0, 2, 3, 6, 12% p/p rispetto alle proteine) sono state aggiunte alla soluzione proteica. Prima del casting, un aliquota di ogni soluzione è stata analizzata per determinare il potenziale zeta e la dimensione delle particelle in soluzione mentre, i film ottenuti sono stati analizzati per le loro proprietà meccaniche. I risultati hanno evidenziato che i valori del potenziale zeta aumentavano linearmente in valore

assoluto quando la quantità di nanoparticelle aggiunta alla soluzione aumentava fino al 3%. Tale risultato evidenzia un effetto stabilizzante dovuta all'aggiunta alla soluzione delle nanoparticelle che, tuttavia è assente quando si arriva a valori delle nanoparticelle pari al 12%. Tali risultati sono supportati anche dall'aumento delle dimensioni delle particelle che aggregano tra loro e precipitano. Tale effetto può essere attribuito ai fenomeni di nucleazione indotti dalle nanoparticelle così come confermato dall'aumento dell'indice di polidispersione. Lo stesso risultato è stato ottenuto analizzando le proprietà meccaniche dei film.
In conclusione i risultati dimostrano che la massima quantità di nanoparticelle che non provoca destabilizzazione della soluzione proteica, e permette di conseguenza di ottenere film con buone proprietà meccaniche, risulta essere pari 3% p/p di proteine.

Effetto della transglutaminasi sulle proprietà dei film nanorinforzati

Per valutare l'effetto delle nanoparticelle sui film reticolati con la transglutaminasi, le soluzioni proteiche sono state preparate aggiungendo MSN o APTES-MSN a pH 12 prima o dopo trattamento con l'enzima. L'incubazione veniva effettuata portando la soluzione proteica a pH 8, e aggiungendo 20 U di enzima/mg a 25 °C per un'ora. Al termine dell'incubazione, la miscela era portata a pH 12, addizionata con glicerolo 42 mM e lasciata in agitazione per 15 minuti prima del casting. I campioni di controllo venivano preparati in assenza dell'enzima.
I risultati hanno evidenziato che l'uso della transglutaminasi permette di ottenere fim con migliori proprietà quando le nanoparticelle sono state aggiunte prima dell'azione dell'enzima, anche se la reticolazione contrastava l'effetto migliorativo sulle proprietà meccaniche che si evidenziava quando venivano aggiunte le sole nanoparticelle. Probabilmente, la formazione dei legami crociati isopeptidici porta all'irrigidimento della matrice proteica modificando di conseguenza le interazioni tra le molecole proteiche e le nanoparticelle. È noto infatti che le proprietà meccaniche dei biomateriali sono largamente associate alla distribuzione e alla densità delle interazioni intermolecolari e intramolecolari dei biopolimeri che determinano la struttura del materiale.

Analisi morfologica

I film ottenuti sono stati analizzati mediante microscopia a scansione e forza atomica al fine di valutare la struttura del materiale sia a livello della superfice che all'interno dei film. I risultati hanno evidenziato che la presenza delle nanoparticelle e la contemporanea reticolazione ad opera della transglutaminasi porta alla formazione di una struttura molto più compatta e omogenea del controllo. Infatti, analizzando in particolare la sezione dei film si evidenzia che la reticolazione della matrice proteica permetteva di inglobare le nanoparticelle portando ad una loro distribuzione omogenea all'interno della matrice proteica. Tali risultati sono stati confermati anche mediante analisi di microscopia a forza atomica che ha evidenziato una riduzione della rugosità della superfice nei campioni contenenti le nanoparticelle e trattati con l'enzima.

Proprietà termiche

Anche gli studi termoanalitici evidenziano che la presenza delle nanoparticelle modifica la struttura del materiale e che questo effetto è molto più evidente quando si utilizzano le APTES-MSN probabilmente a causa delle interazioni ioniche proteina-APTES-MSN che a pH 7 risultano, rispettivamente, caricati negativamente (proteine) e positivamente (APTES-MSN).

Proprietà antimicrobiche a antifungine

Come precedentemente riportato, l'obiettivo di questa tesi era quello di preparare film edibil nanorinforzati aventi attività biologica. A tale scopo le soluzioni proteiche contenenti le nanoparticelle, e reticolate con la transglutaminasi, sono state addizionate con la nisina, un agente antimicrobico classificato come GRAS. Per valutare se la nisina conservava la sua attività biologica all'interno dei film, sia la soluzione proteica, addizionata o meno della nisina, che i relativi film derivati sono stati analizzati mediante il test microbiologico della diffusione in agar, utilizzando il *Micrococcus luteus*.

Per quanto riguarda i test di diffusione in agar delle soluzioni proteiche, non sono state evidenziate differenze significative sulle dimensioni dell'alone di inibizione delle soluzioni contenenti o meno sia MSN che APTES-MSN (3% p/p di proteine), in presenza o assenza della transglutaminasi (20 U/g di proteine), rispetto a una soluzione acquosa contenente la stessa concentrazione di nisina. Questo risultato conferma che la soluzione proteica non aveva un azione inibente sull'attività antimicrobica della nisina. Tuttavia, la comparazione tra la soluzione senza nisina e quella con la nisina evidenziava un attività antimicrobica significativa "intrinseca" della soluzione proteica. Lo stesso risultato veniva osservato analizzando l'alone di inibizione dei rispettivi film. Infatti, i film ottenuti sia in assenza che presenza di nanoparticelle e/o transglutaminasi presentavano una attività antimicrobica intrinseca che migliorava con l'aggiunta di nisina (da 10 a 16 mm). Questo risultato conferma che l'utilizzo di MSN o APTES-MSN, con e senza enzima, non interferiva con l'attività antimicrobica della nisina.

Inoltre sono stati effettuati anche studi sulla attività antifungina dei film rinforzati o meno con le nanoparticelle e la transglutaminasi, attraverso un metodo standard che utilizza come inoculo *Aspergillus niger, Penicillium pinophilum, Chaetomium globosum* ed *Aureobasidium pullulans* e, come controllo, carta da filtro. Film del diametro di 20-25 mm sono stati posizionati sull'agar e la superfice è stata inoculata spruzzando la sospensione di spore con un atomizzatore sterilizzato ad una pressione dell'aria pari a 110 Kpa, in modo che l'intera superficie fosse inumidita. I campioni sono stati incubati per un minimo di 10 giorni e la crescita era monitorata ogni 24 h. I risultati ottenuti hanno evidenziato che mentre sulla carta da filtro si otteneva un'alta crescita di funghi dopo il terzo giorno, nei film contenenti solo proteine la crescita dei funghi iniziava solo dopo 5 giorni, probabilmente a causa dell'attività antifungina "intrinseca" del film. Tale attività veniva significativamente migliorata nei film contenenti nisina, i quali erano in grado di bloccare completamente la crescita dei funghi per almeno dieci giorni.

3. Conclusioni

In questi tre anni di ricerca i risultati ottenuti hanno portato alla conclusione che sia possibile preparare film edibili attivi utilizzando proteine estratte dai semi di veccia amara *(VIcia ervilia),* nanorinforzati mediante incorporazione di nanoparticelle di silica mesoporosa e reticolati attraverso l'azione catalitica della transglutaminasi, a cui possono essere aggiunti additivi biologicamente attivi quali l'oligopeptide nisina dotato di attività antimicrobica e antifungina.
L'aggiunta delle nanoparticelle di silica mesoporosa portano al miglioramento delle proprietà fisico-chimiche dei film proteici ottenuti, in quanto rafforzano la rete proteica e migliorano le loro proprietà meccaniche e di barriera ai gas ed al vapore acqueo. La formazione di legami isopeptidici catalizzata dalla transglutaminasi all'interno della matrice proteica dei film contenenti MSN o APTES-MSN, anche se contrastava il miglioramento delle proprietà meccaniche determinato dalle nanoparticelle, si è mostrata in grado di aumentare ulteriormente le proprietà di barriera dei film rinforzati sia con le MSNs che con le APTES-MSNs. Tale effetto è risultato essere molto più marcato nei film contenenti APTES-MSNs probabilmente a causa delle interazioni ioniche instaurate tra i gruppi amminici carichi positivamente della nanoparticella e le cariche negative presenti sulle proteine della matrice.
Per quanto riguarda l'utilizzo come carrier di composti bioattivi dei film nanorinforzati, i risultati hanno dimostrato che, utilizzando la nisina, non si evidenziano effetti inibenti sull'attività antimicrobica della nisina ad opera della soluzione proteica contenente o meno le nanoparticelle o la transglutaminasi. Inoltre, i film proteici hanno dimostrato di possedere di per sé una significativa attività antimicrobica e antifungina sia in assenza che in presenza di MSNs, APTES-MSNs e transglutaminasi.
In conclusione, anche dagli studi oggetto della presente tesi, l'uso delle nanotecnologia si conferma come uno strumento utile per migliorare le proprietà di alcuni biomateriali di possibile applicazione nel settore alimentare potendo dare origine a film biologicamente "attivi" per l'estensione della shelf life dei prodotti e il controllo dei processi degradativi delle matrici alimentari.

1. INTRODUCTION

1.1 Scientific context

During the last twenty years, researchers are looking for different alternatives to plastic materials. In fact, the environmental impact of plastic wastes is escalating rising widespread global concern and disposal systems are absolutely inadequate. Incineration may engender toxic waste pollution, suitable landfills are limited, and reutilizing techniques for waste are usually expensive and involve high-energy consumption. Adding to it, the petroleum resources are finite and rationed. It is crucial, thus, to find enduring plastic alternatives, especially in short-term food packaging and disposable applications. The most attractive solution to this huge problem is represented by the production of alternative, bio-based and biodegradable/edible, plastics named “bioplastics” (Falguera et al., 2011; Pathak et al., 2014).

This type of biomaterials can satisfactory reached mechanical features and is able to prevent moisture loss and control gas exchanges -such as oxygen, carbon dioxide and ethylene- involved in “respiration” processes of numerous coated food products. The source of most consumed foods is coming directly from nature, where many of them can be immediately eaten as we take them from the tree or ground. However, by increasing of some elements like transportation and distribution systems, the storage needs, and advent of even larger supermarkets and warehouse stores, the majority of foods are not consumed in the farmhouse or close to processing facilities. Therefore, it takes enough considerable time such a food product to reach on the consumer´s table.

During time-consuming steps of handling, storage and transportation, food products start to dehydrate, deteriorate, and lose their appearance, flavour and nutritional value. If no special protection is provided, damage can occur within hours or days, even if this damage is not immediately visible.

The development of biopolymer films has increased the amount of research on biodegradable/edible packaging. Film-forming biopolymers can be proteins, polysaccharides or lipids (Han, 2006). Protein and polysaccharide films can enhance food quality by acting as barriers and by providing protection to a food product after the primary package is opened (Wang *et al*., 2009). These films can be also used for individual packaging of small portions of food, in particular products that are currently not individually packaged for practical reasons.

1.2 Edible films

An edible film or coating may also provide some mechanical protection properties for a food, reducing breakage and improving its integrity. In addition, edible films and coatings are not necessarily intended to eliminate the protective packaging, but they could be also intended to interact with the conventional packaging to enhance the shelf life and the product quality. When the edible film or the coating prevent or modulates the exchanges of moisture, oxygen, carbon dioxide, aromas or oil

between the food and the environment, the quality and the shelf life of the food also increased.

Therefore, the advantages of the biodegradable/edible films and coatings are the following:

- They are produced from renewable materials
- They contribute in reducing environmental pollution
- They can be consumed with the packaged product
- They can be tailored to prevent deterioration and inter-component moisture and solute migration in foods.

On the other hand, the actual disadvantages are on:

- Their poor mechanical and barrier properties compared with the conventional plastics
- Their lower production
- Their higher price, comparing to synthetic plastics.

However, it should be noted that the synthetic plastics pollution is an expensive matter, the environmental damage costs having been estimated over $13 billion/year (United nation agency, 2014). The edible films might overcome this drawback and, thus, in the long run bioplastics production may become cheaper comparing with conventional plastics.

Components used for the preparation of biodegradable/edible films can be classified into two categories: hydrocolloids (such as proteins or polysaccharides) and lipids (such as fatty acids, acylglycerol, waxes and surfactants). These biopolymers could be used alone to make protein-, polysaccharide- and lipid-based films, or in combination to make hydrocolloid blended films (protein/polysaccharide-based films) or composites (hydrocolloid/lipid-based films) (Song and Zheng, 2014). Polysaccharides used for edible films or coatings include cellulose, starch, pectin, seaweed extracts, exudate gums, microbial fermented gums, chitosan or their derivatives. Edible films and coatings, prepared from polysaccharides, proteins and lipids are environmentally friendly and have a variety of advantages over synthetic materials, such as biodegradability, edibility and biocompatibility.

Moisture barrier properties of polysaccharide-based films are poor and the addition of proteins can provide films with improved characteristics not only in terms of acceptable barrier capacity to water vapor but also in terms of higher mechanical resistance. The implement of these features can be further enhanced by enzymatic reticulation via transglutaminase, an enzyme able to crosslink proteins by the formation of covalent isopeptide bonds between the side chains of specific glutamine and lysine residues occurring in the polypeptide sequences (Porta, 2011). Moreover, for the preparation of the edible films different plasticizers are added to improve the flexibility and handling of the biomaterial as well as to hinder such as cracking. Even though the plasticizer used during the research material of my project is glycerol, the investigations of natural and biodegradable innovative plasticizers with a lower toxicity and a better compatibility according to several plastics, by substitution of

conventional plasticizers such as glycerol or sorbitol, have become attracted to a market according with an increasing worldwide trend towards the use of biopolymers (Vieira et al., 2011).
Edible films and coatings can be obtained in various ways such us spraying, dipping or casting. Spraying is used to apply a uniform coating over a food surface, and is a potentially a more controllable method of coating. However, spray-coating requires that the bottom surface of the product be coated in a separate operation after application of the initial coating and drying (Dangaran et al., 2009).
Dipping is advantageous when a product requires several applications of a coating to obtain uniformity on an irregular surface (Embuscado and Huber, 2008).
Casting Films obtained through evaporation were found to have lower water vapor permeability than those prepared by spraying (Pickard et al., 1972).

1.3 BV protein

Bitter vetch is an ancient grain legume crop originated in the Mediterranean region that can be found today in many countries around the world. This annual *Vicia* genus shows several favourable features, such as having 63 high yields and being a cheap protein source resistant to drought and insects.
Therefore, bitter vetch's tubers, which are quite sweet and nutty-tasting, have undoubtedly been eaten in their time, is widely cultivated for forage and seed yield because of its high nutritional value, capacity of nitrogen fixation and ability to grow in poor soils.

Figure 1. *BV*. Plant (left) and seeds (right)

Bitter vetch is widely cultivated for forage and seed yield because of its high nutritional value, capacity of nitrogen fixation and ability to grow in poor soils (López Bellido, 1994; Sadeghi et al, 2009).
BV were recently analyzed as sustainable alternative source to produce biodegradable films, edible coatings and, potentially, properly shaped biodegradable containers (Arabestani et al., 2013).

1.4 Glycerol

The addition of plasticizers to improve the mechanical properties of edible films is highly required, and various plasticizers, usually polyols, have been employed to increase the flexibility and workability of edible films. Among the plasticizers, glycerol is one of the most broadly used in film-making techniques, and it has been successfully employed in the production of protein edible films.
Glycerol, as plasticizer, is very widespread into most edible films. A plasticizer is defined as substantially non-volatile, high boiling, non-separating substance, which when added to another material changes the physical and/or mechanical properties of that material (Banker, 1966). It is a water-soluble, polar, non-volatile, and protein miscible. These properties make glycerol a suitable plasticizer for use with a compatible water-soluble protein.

1.5 Reinforced edible films

Due to their usually poor mechanical and barrier properties compared to the synthetic polymers, the use of edible films has been limited until now. One way to overcome these drawbacks for the edible film and bring them to the top of the conventional plastics is on the reinforcing with different materials that allow a better improvement for their physico-chemical properties (Azeredo et al., 2009).
Therefore, the bio-functional properties of biodegradable/edible films can be modified by incorporating further active compounds into the matrix. However, the behavior of the active compounds into a film matrix, is generally modulated by physical, chemical and biological properties of the matrix structure, which, in turn, are depending on its size and morphology at nano-scale level. For these reasons, different studies have been focused to the reinforcement of edible films by nano-materials able to act also as nano-containers for active compounds (Hang et al., 2012; Voon et al., 2012). Hence, these nanomaterials, may improve both the mechanical and the permeability properties of the biodegradable/edible films (George and Siddaramaiah. 2012).

1.6 Nanotechnology

The potential benefits of nanotechnology have been recognized by many industries, and commercial products are already being manufactured mostly in the microelectronics, aerospace, and pharmaceutical industries. (Campos et al., 2011). Developments in these industries are guided by fundamental and applied research in domains such as physics, chemistry, biology, engineering, and materials science. In contrast, the applications of nanotechnology within the food industry have been so far rather limited (Weiss et al., 2006). Nowadays, this trend is changing very fast, being nanotechnology a fundamental part of the food industry (Rashidi and Khosravi-Darani, 2011).
Nanotechnology is a science that has been studied for decades but, since its beginning, the instrumental inability to put in evidence the NPs presented a great

drawback of this scientific issue. With the development of advanced microscopy devices, nanotechnology has evolved considerably in the last fifty years. In fact, the ability to observe particles at the nanoscopic level has opened up a world of possibilities within science and industry. Therefore, nanotechnology is becoming increasingly important especially for the food industrial sector undergoing a rapid development (Duran et al., 2013). The association between the small particle size and large surface areas leads to NPs possessing unique features and, multiple potential applications as main resultant. Structures on this scale level have been proved to have unique and novel functional properties. Due to its interdisciplinary research capabilities in different branches of science like biology, chemistry, engineering and physics, nanotechnology increased the developing by new materials (Arora et al., 2010).
Promising results have been obtained and some applications have been already carried out in the field of nutrient delivery systems through bioactive compound nano-encapsulation, as well as biosensor one, with the aim to detect and quantify pathogens, several chemicals or food composition alterations (Neethirajan and Jayas., 2011). The use of NPs in the development of nanocomposite materials represents a new strategy to improve physical properties of several polymers, including mechanical strength, thermal stability and barrier properties. Furthermore, since nanotechnology is a science in continuous advancement, food industry will benefit from it mainly through the production of innovative active and intelligent packaging (Mihindukulasuriya and Lim., 2014).

1.6.1 MSN

Over the last decades, MSNs, components belonging to one of the most important porous material, have been widely used due to their unique features, such as high surface area, controllable pore structure, large pore volume, optically transparent properties, low toxicity, high chemical and thermal stability, and versatile chemical modifiable surface (Liu et al., 2016). The main fields of MSN applications have been:

- Release system for drug delivery system (Yonchevaa et al., 2014)
- Adsorbent for bioactive compounds (Cotea et al., 2014)
- Development of cancer therapies (Feng et al., 2016)
- Molecular imaging (Sixiang et al., 2013)
- Food industry (Bernardos et all., 2013)
- Catalysis (Yang et al., 2012)
- Optical devices (Lee et al., 2010)
- Polymer filler (Klober et al., 2008)

MSN (MCM-41) synthesis occurs by using cetyltrimethylammonium bromide (CTAB) micelles as a surfactant. The addition of tetraethylortosilicate (TeOS) creates the

micellar rod. Finally, the obtained solid is calcinated to take off the surfactant (Figure 2).

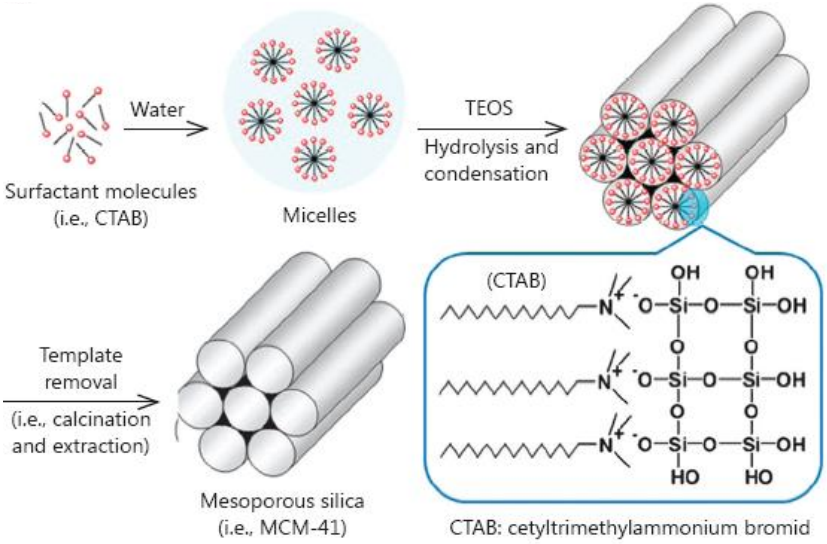


Figure 2. MSNs synthesis (Yang et al., 2014)

One important possibility to functionalize MSNs, shown in Figure 3, represents the substitution of hydroxyl- groups with different amino- groups, such as APTES.

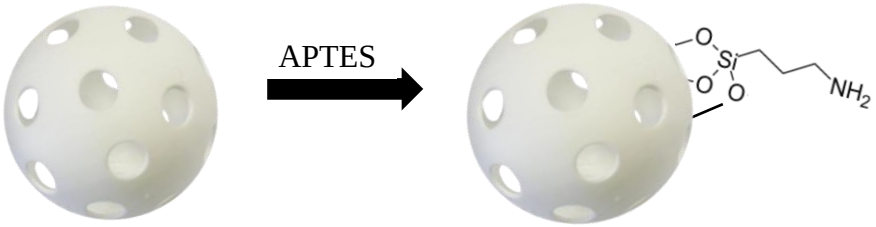


Figure 3. APTES-MSN synthesis

1.6.2 Biopolymers/nanoclay composites

Edible films can be innovatively modified by nanoparticle (NP) insertion to create a nanocomposite where the edible films are used as matrix reinforced with different fillers, like TiO_2, SiO_2, Carbon nanotubes or MSNs (Bilbao Sainz et al., 2010). The nanocomposite preparation conditions can affect the arrangement between polymer and nanoclay.

The pH that can affect the loading of possible radicals that are in the chains and the nearer or awayer proximity to the isoelectric point (pI) is also a parameter to be considered. The temperature is other determination factor, as well as the holding time able to influence the ordering of the structure. The solution stirring and the periods before and after the addition process can greatly conditioned the

handling/performance. Therefore, all these factors may influence the morphology of the final structure produced by the interaction between the biopolymer(s) and the nanoclay (Sothornovit et al., 2009).
Figure 4 shows an exfoliated (a), intercalated (b) or intercalated/flocculated (c) morphology of the biomaterial, being the intercalated one the optimum option to obtain a more compact structure. Therefore, the production of nanocomposites represents a new possible alternative to the conventional technologies for improving bioplastics mechanical and barrier properties (Arora et al., 2010).

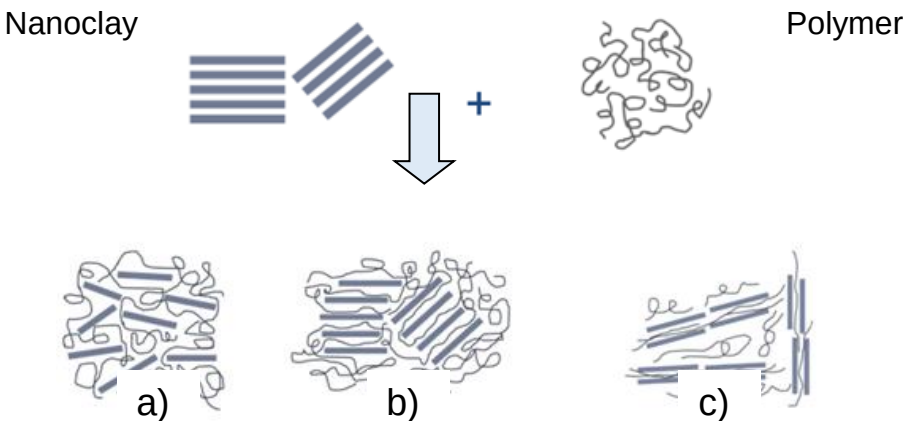


Figure 4. Possible biopolymer/nanoclay complex morphologies. a) exfoliated; b) intercalated; c)intercalated/flocculated. (Galimberti, 2011)

1.6.3 mTGase-containing films

The reinforcement of protein-based films can be further obtained by enzymatic protein reticulation via transglutaminase (EC 2.3.2.13), an enzyme belonging to the class of transferases (Marx et al., 2008; Trespalacios and Pla, 2007) and able to crosslink proteins by the formation of covalent isopeptide bonds between the side chains of specific glutamine and lysine residues occurring in the polypeptide sequences (Porta, 2011). Figure 5 exposes the transamidation reaction occurs when the acyl acceptor is the ε-amino group of an endoprotein lysine residue and the acyl donor is the ɣ-carboxamide group of an endoprotein glutamine residue occurring either in the same (panel A) or in a different (panel B) polypeptide chain. For the first case an intra-molecular crosslink is obtained, whereas for the second one the preaction product is an inter-molecular crosslink.

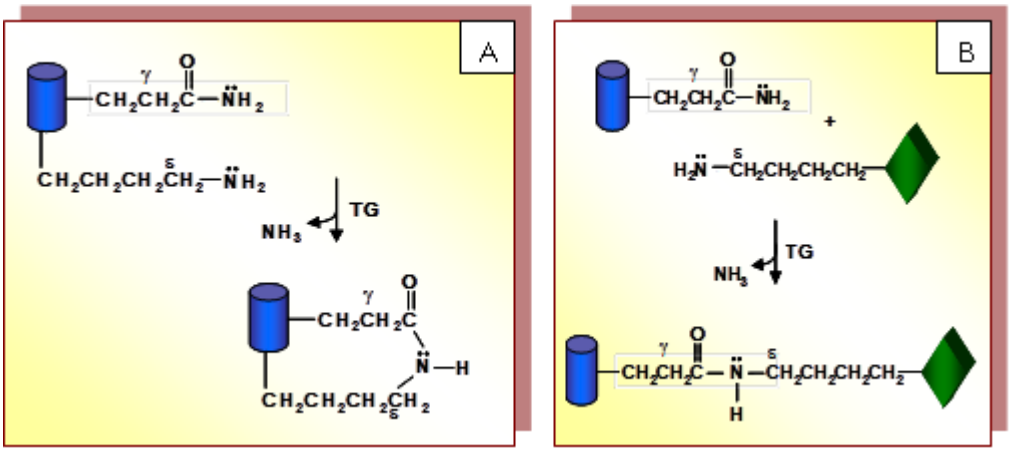


Figure 5. mTGase catalyzed reactions

In 1989 mTGase was isolated from *Streptoverticillium* sp. and its characterization lead to its isoform could be extremely useful as a biotechnological tool (Ando *et al*., 1989). mTGase was proven to be:

- *Calcium independent* (Motokia and Segurob, 1998)
- *Active in a wide range of pH (4-9)* (Kieliszek and Misiewicz, 2013)
- *Resistant between 4-60°C (Ho et al., 2000)*
- *Commercially available* (Zhu et al., 1995)
- *Food grade (Yokoyama et al., 2004)*
- *Useful to modify protein structure and biological properties* (Singh Gujral and M. Rosell, 2004)

The mTGase active centre components belong to cysteine, histidine and either asparagine or aspartate residues.
Different researchers teams (Shimba et al. 2002; Washizu et al. 1994; Ando et al. 1989) demonstrated mTGase possess a different reactivity to some food proteins, this feature representing a very useful tool to modify the functionality of proteins in various food products. mTGase is also known to be inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} ions, which bind to the thiol group of cysteine in the active centre (Macedo et al.2007, Ando et al. 1989).
Since 1957, when there is attested a first description belonging to the Clark´s team (Clarke *et al*. 1957) for a transamidating activity in guinea pig liver, a research activity on this class of enzyme has been growing, beginning with human health (biomedicine and cosmetics) and continuing with industrial applications in different fields (food, leather and textile industries). Many studies were focused on the impact of mTGase-induced protein crosslinking on dairy products providing from cow milk

(Lauber et al., 2000; Bonisch et al., 2007; Jaros et al., 2007), whereas only a few data are available for goat milk (Farnsworth et al., 2006; Rodriguez Nogales, 2006). Today, mTGase have proved to be one of the most important industrial enzymes that accounted for around 30% of the world production enzymes with a wide variety of applications (Aidaroos et al., 2011). As an overlooking to protein-based edible films, mTGase represents one of the most used crosslinking agent, being able to improve film barrier properties and mechanical strength.

1.7 Antimicrobial film and antifungal additives

Edible films and coatings could carry preservatives with the aim of improving the microbial surface stability of foods. Microbial contamination primarily occur on the food surface, due to its post-processing handling. The use of active packaging films containing antimicrobial agents could be an efficient procedure able to slow down the migration of the agents from the packaging material to the food product´s surface (Pérez-Pérez, 2006). Therefore, antimicrobial packaging is a form of active packaging that could extend the shelf-life of the products providing microbial safety for consumers (Rooney, 1995). The development of antimicrobial packaging technologies could play an important role in extending the shelf-life of the food and reducing the risk from pathogens (Appendini and Hotchkiss, 2002).
The most common used antimicrobials agents for edible films are represented by: organic acids, the polysaccharide- derivative chitosan, the polypeptide nisin, the lactoperoxidase system and some plant extracts and essential oils. A prerequisite to include a compound into the antimicrobial agent class, so to be added to edible films, will focus on its effectiveness against microorganisms *in vitro* and on its possible interactions with the film forming solution (FFS) components.

Nisin

Nisin is the antimicrobial agent used in the experiments carried out in the present project. It is a natural antibiotic obtained from the bacterium *Lactococcus lactis*, (Perin et al., 2012). It has a molecular mass of 3.4 kDa and is composed of 34 residues of amino acid. It is normally present in dairy products and is used to prevent the development of fungi, spores, and microbes (Schillinger et al, 1997). Nisin is considered safe by the FDA and received GRAS designation in 1988. Its activity is measured in International Units (IU), and approximately 1 μg of pure nisin corresponds to 40 IU (Rossi-Marquez, 2009). Diffusion agar method is the most common used way to determine the antimicrobial activity of the peptide. The antimicrobial diffuses from the disk to the culture medium inoculated with certain microorganisms, will produce a zone of inhibition (Davidson and Branen, 2004).
The harmlessness of nisin for humans and its rapid enzymatic proteolysis in both stomach and intestinal tract explains its widespread use in many countries, including those with the most stringent regulations for food additives such as European Union and the United States. The use of nisin as food preservative "should be considered

acceptable being intake average daily 0-33,000 U/kg" (WHO, 1969). From 100 to 400 units per gram of food (or 2.5-10 ppm) are recommended for the conservation of food. Nisin was used for the first time in the ´50 to inhibit cheese microbial spoilage (García et al., 2010). From then, it was used in numerous thermal food processes, applications for canned food, various dairy products, liquid egg, pasteurized and/or flavoured milk, beverages, fermented products, meat, instant soups, and food of plant origin (Guerra et al., 2005).

The use of nisin as food conservative allows the decrease of temperatures during heat treatments, as well as the times of these treatments, allowing a saving in the consumption of energy in the process, improves food nutritional value, appearance, flavor and texture, greatly increasing the shelf life of the products (Guilbert et al., 1996). It is worthy to note that nisin is the only bacteriocin approved as preservative food, which explains the huge growth of its use in the food industry. This use is governed by the “FAO/WHO Codex Committee on Milk and Milk Products” which accepted nisin as a food additive in a concentration not exceeding 12.5 mg/Kg (Rossi et al., 2009).

The nisin antimicrobial activity is directly related to its solubility and structural stability that depends on pH value. Its solubility is higher at acidic values pHs: 12% at pH 2.5 and 4% at pH 4, and the case whereas nisin is almost insoluble at neutral pH. Similarly, its antimicrobial activity is higher at acidic pHs and gradually decreases the increasing of pH value. This feature may be explained by an irreversible modification of its structure (Hurst, 1981). The exposition temperature is also an important factor that dramatically influences the effectiveness of the peptide (Cruz-Chamorro et al., 2006).

Nisin is generally an effective agent against of a wide bacteria spectrum such as *Listeria monocytogenes* and other Gram positive microorganisms (Gharsallaoui, 2016), and particularly against of the situations that produce heat-resistant spores. It inhibits specific strains of pathogens in the food, such as *Clostridium spp., Clostridium botulinum, Staphylococcus aureus, Streptococcus hemolyticus, Listeria monocytogenes, and Bacillus stearotermophilus*. It has also an effect against of pathogenic Gram negative bacteria, such as *Escherichia coli* and *Salmonella* and, by combined with chelating agents, such as EDTA, can cause the alteration of the cell wall promoting the contact with the cytoplasmic membrane (Belfiore et al., 2007).

Figure 5 shows a schematic representation for the mechanism of nisin activity (Breuknik and de Kruijff, 2006). First, nisin reaches the bacterial plasma membrane (a), where it binds to Lipid II via two of its amino-terminal rings (b). This is then followed by pore formation (c), which involves a stable transmembrane orientation of nisin. During or after assembly of four 2:1 (nisin: Lipid II) complexes, four additional nisin molecules are recruited to form the pore complex (d).

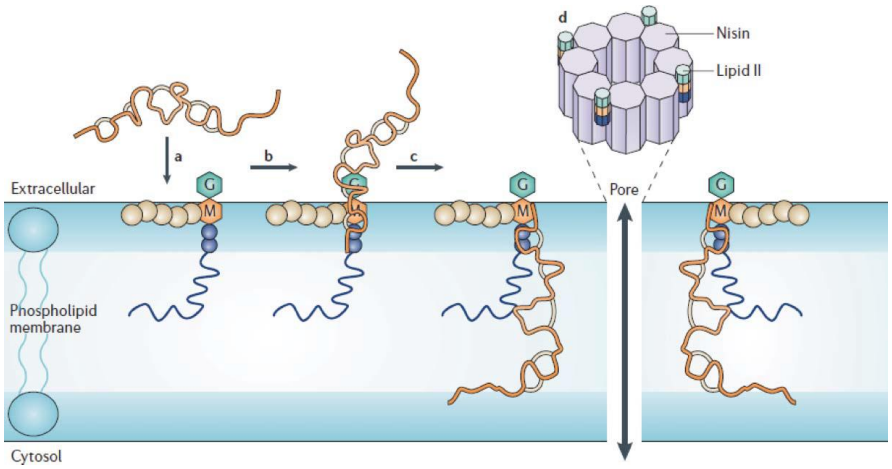


Figure 6. Molecular mechanism of nisin (Gharsallaoui, 2016)

Nisin is used in a high variety of food products, both single or in combination with other conservatives, such as benzoic acid or sorbic acid. Due to its good solubility in aqueous media it can be previously suspended in pasteurized solution of water or milk and applied subsequently to the thermally treated foods. It can also be dispensed as a dried powder.

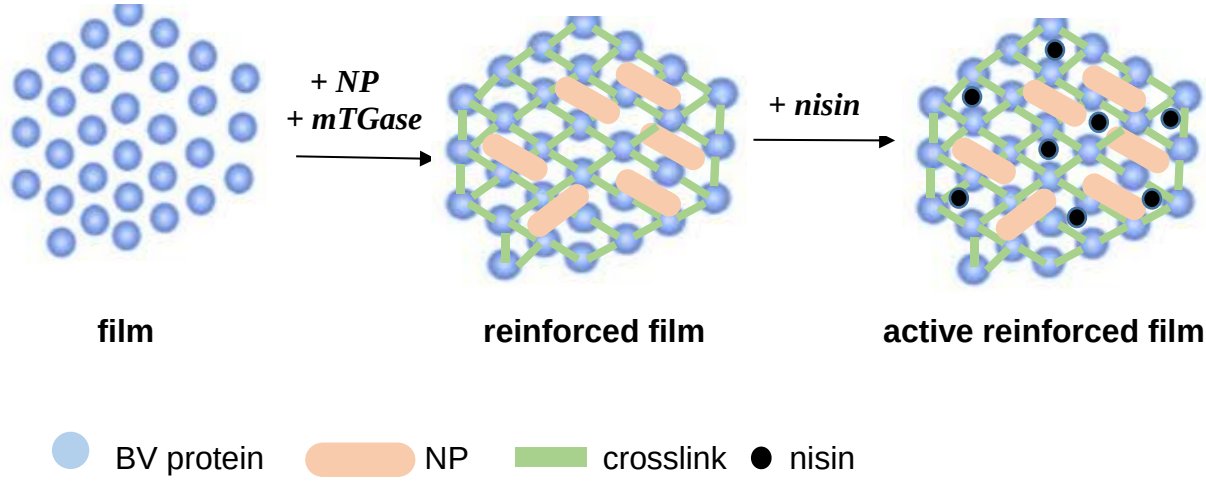
Tipically, the suggested dosage varies in a range between 10 and 500 mg/Kg or mg/L food (Govaris et al., 2010). Something else is only a guide, the precise dose depending on the nature of the food, the processing conditions, the microbiological load and the shelf life specific requirements. In addition, nisin can be stored at room temperature, being stable for two years starting from the date of processing after storage in the original container in dry conditions and away from direct light direct at temperatures between 4 and 10 °C (Solomakos et al., 2008).

Further experiments will be carried out by preparing edible films containing antifungal additives to test prove capability of protein-based coatings to be used as defense tool against different microorganisms that negatively affect food storage and shelf-life.

1.8 General objective of the experimental research

The main objective of this thesis was the preparation of a protein-based edible film, reinforced by the addition of different NPs and the action of a crosslinking enzyme, to improve the physico-chemical properties of a potential innovative coating. The film exhibiting the best mechanical and barrier properties was then added with an antimicrobial/antifungal preservative to realize an active food packaging. To this aim, a protein concentrate extracted from bitter vetch (BV, *Vicia ervilia*) seeds was used as renewable resource to obtain the basic matrix of hydrocolloid biodegradable/edible films. BV proteins were chosen as film matrix source for the following reasons: i) high seed protein concentration; ii) low plant cultivation cost; iii) high yield of production and iv) possibility to grow in marginal soil. MSNs and APTES-MSNs were added to the BV protein-based FFS to obtain reinforced nanocomposite materials, whereas mTGase was used as crosslinking agent to better

stabilize the nanoparticles into the protein network. Finally, nisin was used as antimicrobial/antifungal additive to realize a potential active food coating.



2. *MATERIALS AND METHODS*

2.1 Materials

BV seeds were obtained from a local market in Gallicchio (PZ), Italy. BV protein concentrate (BVPC) (78% of proteins determined by the Kjeldahl's method) was prepared as previously described (Sabbah et al., 2016). mTGase from *Streptoverticillium* sp. (Activa WM; specific activity 90 units/g) was supplied by Prodotti Gianni SpA (Milano, Italy). The enzyme solution was prepared by dissolving the commercial preparation in distilled water at a concentration of 100 mg/mL and the mixture was centrifuged at 10,000×g for 2 min to remove the precipitate. Estimation of enzymatic activity was carried out by a colorimetric hydroxamate assay (Pasternack et al., 1998). TeOS (98%), APTES, CTAB and glycerol were obtained from Sigma (Steinheim, Germany). Casein derived peptone and yeast extract were from BD chemical (Greenwood Village, USA), whereas meat extract was obtained from Fluka. All other chemicals and solvents used in this study were analytical grade commercial products.

MSNs were synthesized, as reported by Chen et al. (2011), at the Complutense University of Madrid (Spain) during my first stay on September 2015. CTAB (0.5 g), used as the surfactant, was dispersed in 240 ml of water and 1.75 ml of 2 N NaOH at room temperature by using an ultrasonic bath. The mixture was heated at 80 ºC and then 2.5 ml of TeOS were added drop by drop in 5 min. The solution was vigorously stirred for 2 h at 80 ºC. The solid products were collected by filtration, washed twice with distilled water, twice with ethanol, and finally dried at room temperature. The resulting powders were calcinated at 500 ºC for 5 h to remove the surfactant. The functionalization of MSN was achieved by suspending 0.5 g MSN in 10 mL of ethanol after that APTES solution (100 mg/mL) was added in a proportion of 1mL/100 mg of MSN. The final mixture was stirred overnight at room temperature. The APTES functionalized MSN (APTES-MSN) was collected by filtration and dried after two times ethanol washing.

2.2 NP characterization

The elemental analysis (C, H and N %) of both MSNs and APTES-MSNs was carried out by LECO® CHNS-932. Bulk samples are freeze-dried, crushed and homogenized using a mortar and pestle or electric mill, and weighed into a tin sample cup (crucible) with vanadium pentoxide catalyst. The crucibles are then closed (referred to as "wrapping" the sample) and placed in the autosampler for instrumental analysis.

X-ray scattering experiments were performed using synchrotron radiation as an X-ray source with a long-slit collimation system at. The incident X-ray wavelength λ was 0.154 nm, and the cattering angle 2θ was from 2 to 35º with steps of 0.02º and a counting time of 0.8 s/step. The scattered X-ray intensities were recorded using imaging plate technology. The air scattering and the absorption of the sample were corrected. For as-made samples, XRD peaks were observed in the interval of d-spacings up to 10 Å, which comprised 40 symmetrically independent reflections.

Nitrogen adsorption/desorption isotherms of NP samples were obtained using a volumetric adsorption analyzer (Model Autosorb-1, Quantachrome Instrument, Boyton Beach, FL) at 77 K. Powdered samples of 50−100 mg were degassed at 90 °C until a pressure of 10 μm Hg was reached for 1 h and then at 120 °C for 6 h prior to measurement. Total pore volume was estimated from the amount adsorbed at 0.99 relative pressures.

$$SA_{BET} = \frac{CSA \times N_A}{22414 \times 10^{18} \times (S + Y_{INT})}$$

Where SA_{BET} is the BET surface area (m^2/g); CSA is the analysis gas molecular cross-sectional area (0.162 nm^2 for N_2); N_A is the Avogadro constant 6.023 10^{23}; S is the slope (g/cm^3); YINT is the Y-intercept (g/cm^3).
Fourier Transform Infrared (FT-IR) spectra of the samples in KBr pellets were measured using Bruker Vertex 80 V FT-IR spectrometer with at least 16 scans and a resolution of 4 cm^{-1}.
NP pore volume and pore size distributions were obtained from an adsorption branch by using the Barrett, Joyner and Halenda (BJH) method. It is a method to determinate pore size distribution of a mesoporous solid based on the Kelvin equation, where r_p is pore radius (Å).

$$r_p = \frac{4.15}{\log \frac{P_0}{P}} + 3.54 \times \left(\frac{-5}{\ln \frac{P}{P_0}} \right)^{0.333}$$

Finally, the particle size and morphology of the prepared MSNs were characterized by using Technai G2 T20 Transmission Electron Microscope (TEM) (FEI, The Netherlands). The MSNs at a concentration of 1 mg/mL were dispersed in ethanol and sonicated for 30 minutes, and 5 mL of this dispersion was transferred to the TEM grids. The TEM grids were dried overnight at 40 C, and imaged at 200 kV.

2.3 FFS preparation and casting

FFS was prepared by dissolving BVPC in distilled water (10 mg/mL) at pH 12 and then the pH of some FFS samples was adjusted to different values (7 and 10) by adding 1 N HCl. All FFSs were left stirring for 15 min and, at the end, glycerol was added until a final concentration of 42 mM was reached.

2.3.1 Optimal amount of NPs

In order to evaluate the optimal amount of NPs, some FFS samples prepared at pH 12 were mixed with different amount (1, 2, 3, 4, 6 and 12 % w/w of solid in FFS) of

NPs before the addition of glycerol; NPs (10 mg/mL in H_2O) were suspended at pH12 for 5 min by the aid of an ultrasonic batch before their addition to the BVPC FFSs.

2.3.2 Effect of mTGase

To study the effect of mTGase, some FFS samples prepared at pH 7 were treated for 1 hour in the presence of the enzyme and, at the end of incubation, brought at pH 12 before the addition of glycerol.
In order to evaluate the effect of mTGase-mediated crosslinks in the presence or absence of NPs it was prepared two additional FFSs. The first FFS sample was incubated at pH 7 in the presence of mTGase, then brought to pH 12 and finally mixed with NPs. The second FFS sample, prepared at pH 12, was mixed with NPs and, after that the pH was brought to pH 7, it was incubated with mTGase. At the end of incubation the pH of the enzyme containing FFS was newly adjusted to pH 12.

2.3.3 Zeta-potential and particle size

Electrical potential (zeta-potential) and particle size for all the FFS samples prepared were measured using a Zetasizer Nano-ZS (Malvern Instrument Ltd., Worcestershire, UK). Three independent zeta-potential measurements at various pH values were carried out on each sample. 1.0 mL of the solutions were introduced in the measurement vessel. Temperature was set at 25ºC, applied voltage was 200 mV and duration of each analysis was approximately 10 min. The mean hydrodynamic diameter of particles was also determined as a function of pH with the Zetasizer Nano-ZS by using dynamic light scattering. The device uses a helium-neon laser of 4 mW output power operating at the fixed wavelength of 633 nm (wavelength of laser red emission). All the results were reported as mean ± standard deviation.

2.3.4 Casting

Finally, 50 mL of each FFS were casted on polystyrene Petri dishes (150x15 mm) and the films were obtained by drying at 25 °C and 45% RH for 48 h. An aliquot (1 mL) of all the FFSs were saved before casting for zeta-potential and particle size measurements. The obtained films were characterized for their physicochemical, morphological, and biological properties.

2.4 Film morphological characterization

2.4.1 SEM

The backscattered NP electron images were obtained from a JEOL JSM-840A instrument operating at a primary energy of 10 kV. An analysis of the electron microscopy data was performed using the DigitalMicrograph™ (Gatan Inc.) software.

The dried film samples were mounted on aluminium stubs with double-sided adhesive tape, and coated with a thin layer of platinum. Morphological observations of the surface and cross-section (fractured under liquid nitrogen prior to visualization) of the films were done with a scanning electron microscope (Jeol, model JSM- 5800, Tokyo, Japan) at 5-8 kV.

2.4.2 AFM

A Topometrix (Explorer atomic force microscope system (SantaClara, CA) was used for all film analyses. Two types of AFM scanners were used for surface examination of the films. Imaging of areas from 150 μm square to 10 μm square was performed with a tripod, piezoelectric scanner. The highest quality images in this range were obtained in the contact mode. Higher resolution, small area scanning was done using a tube-type scanner with a cylindrical piezoelectric configuration. All imaging was done under ambient (in air) conditions. Noncontact mode scanning was primarily performed using low resonant frequency tips. These tips were found to provide better quality images of the whey protein films than high frequency tips. We also determined that viewing the films from a 3-dimensional perspective provided a topographic detail that could not be seen in the top views.

2.5 Film physicochemical characterization

2.5.1 Thickness measurements

Film thickness was measured with a micrometer model HO62 (Metrocontrol Srl, Casoria (Na), Italy) at five random positions over the film area. Values are mean ± standard deviation (SD) of five replicates.

2.5.2 DSC

The instrument used to determine the film thermoanalytic properties, as well those of MSNs and APTES-MSNs, was a DSC Q 200 V24.11 Build 124. Triplicate sample pans of 5 mg were placed into preweighed sample pans were weighed in a precision balance (Scientech., SA210), were conditioned in hermetic aluminium pans, and heated at 5ºC/min, between room temperature to 200ºC, in inert atmosphere (50mL/min of N_2). The reference was an empty pan. The glass transition temperature (*Tg*) was calculated as the inflexion point of the base line, caused by the discontinuity of the specific heat of the sample and melting point (Tm), as the lowest point of melting of the phase transition. Data analysis of each film was performed with OriginPro 8.6 program (OriginLab, Northampton, MA, U.S.A.).

2.5.3 TGA

TGA was performed on a Mettler Toledo TC15 TA controller over the 30 to 250 °C temperature range at a heating rate of 10 °C/min. The sample weights examined were between 5 and 10 mg. Nitrogen gas flowed over the open crucibles containing the sample as the analysis was performed. The percentage weight loss during the heating cycle was estimated using the associated software (Abugoch et al., 2012).

2.5.4 FT-IR spectroscopy

The analysis of structural links of edible films was performed using FT-IR measurements on a Bruker model IFS 32 spectrometer. Spectra were obtained by averaging 60 scans over the spectral range of 400 to 4000 cm^{-1}. Data analysis of each film was performed with OriginPro 8.6 program (OriginLab, Northampton, MA, U.S.A.).

2.5.5 Mechanical properties

Film tensile strength (TS) and elongation at break (EB) were measured by using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). Film samples were cut, using a sharp razor blade, into 10 mm wide and 40 mm length strips equilibrated overnight at 50% ± 5% RH and 23 ± 2 °C in an environmental chamber. Ten samples of each film type were tested. Tensile properties were measured according to the ASTM (1991) Standard Method D882 using Test Method A, the static weighing, constant rate-of-grip separation test. The crosshead speed was 1 mm/sec in a tension mode.

2.5.6 Barrier properties

Film permeabilities to gas (O_2 and CO_2) and water vapour were determined by using a MultiPerm apparatus (Extrasolution s.r.l., Pisa, Italy). Duplicate samples of each film were conditioned for 2 days at 50% RH before measurement. Aluminium masks were used to reduce film test area to 5 cm^2, whereas the testing was performed at 25 °C under 50% RH.

2.6 FFS and film biological properties

2.6.1 Antimicrobial activity

The films containing APTES-MSN and mTGase were tested as active coatings by adding nisin from 0.01 to 2 IU/mL of FFS before casting. The method used to determine the antimicrobial properties was the agar diffusion test. In this procedure, agar plates are inoculated with a standardized inoculum of the specific microorganism. Generally, antimicrobial agent diffuses into the agar and inhibits

germination and growth of the microorganism and then the diameters of inhibition growth zones are measured. We have used *Micrococcus luteus* (NCBI 8166) a Gram-positive bacteria with a spherical morphology as microorganism. After autoclaving, the assay medium was cooled to about 45°C and then inoculated at a rate of 1% (v/v) with the microorganism (final concentration about 10^7 cfu ml^{-1}). Medium was then dispensed into sterile Petri plates (100~ 15 mm) to appropriate depths. Plates were refrigerated at 4°C for at least 2 h to allow thorough solidification. Test wells were then bored into the agar (6 wells per plate) using a 6 mm diameter glass tube with slight suction applied to remove the agar from the well. For FFS, test solutions (40 µl) were then dispensed into individual wells and plates were incubated at 30°C in a humidity chamber (to prevent desiccation) for 24-48 h. Zones of inhibition were measured with a calliper (Figure 1) to the nearest 0.01 nm. From this data a regression equation was calculated.

Figure 1. Calliper

Film samples were cut using a sharp razor blade into 10 mm diameter and placed on the top of agar with *Micrococcus luteus* and incubated at 30°C for 24 hours. To calculate the inhibition halo diameter the whole zone area was measured and subtracted from the film disc area, this difference was reported as the ‘‘zone of inhibition”. The contact area was also examined visually to evaluate growth inhibition underneath the film disk contain.

2.6.2 Antifungal activity

Film samples were also tested for their antifungal activities by following the antifungal ASTM Standard method (D618 Method of conditioning Plastics and Electrical Insulating Materials for Testing). *Penicillium pinophilum Aspergillus niger, Aureobasidium pullulans* and *Chaetomium globosum* were used by incubating the subcultures at 30 ºC for 15 days and introducing the seeds of the fungi in the agar.
Once the fungi were grown, a spore suspension of each fungus was prepared by pouring into each subculture 1 mL of an aqueous sterile solution containing 0.05 g/L of sodium dioctylsulfosuccinate (Tween 80), a nontoxic wetting agent. Spores were washed three times and then were diluted with sterile nutrient-salts solution to obtain a suspension containing 1.000.000 ± 200.000 spores/mL, as determined by an optical microscope with a Neubauer chamber. Film samples were cut, using a sharp razor blade, into 10 mm diameter pieces and placed on the top of agar (Potato

dextrose agar, PDA) in Petri dishes (90x20 mm). Film surfaces were inoculated with the composite spore suspension by spraying the suspension from a sterilized atomizer with 110 kPa (16 psi) of air pressure until the entire surface was moistened. The inoculated test specimens were covered and incubated at 30 ºC and 85% RH for 10 days, recording the fungal growth in the films each day.

2.7 Statistical analysis

JMP software 8.0 (SAS Campus Drive, Building S, Cary, NC) was used for all statistical analyses. The data were subjected to analysis of variance, and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $p<0.05$

3. RESULTS AND DISCUSSION

3.1. Preparation and characterization of NPs

3.1.1 MSN

In order to prepare BVPC reinforced edible films we have synthetized MSNs. The starting nanoparticles (a calcined MCM41-like solid) were synthesized by alkaline hydrolysis of TeOS as inorganic precursor in the presence of the cationic surfactant CTAB as porogen species (Zhao et al., 2009). The obtained MSNs, analyzed by TEM (Figure 1A), present a spherical geometry with homogenous size distribution (Figure 1B) with an average size of 143 ± 26 nm and an MCM-41 type channel-like mesoporous structure.

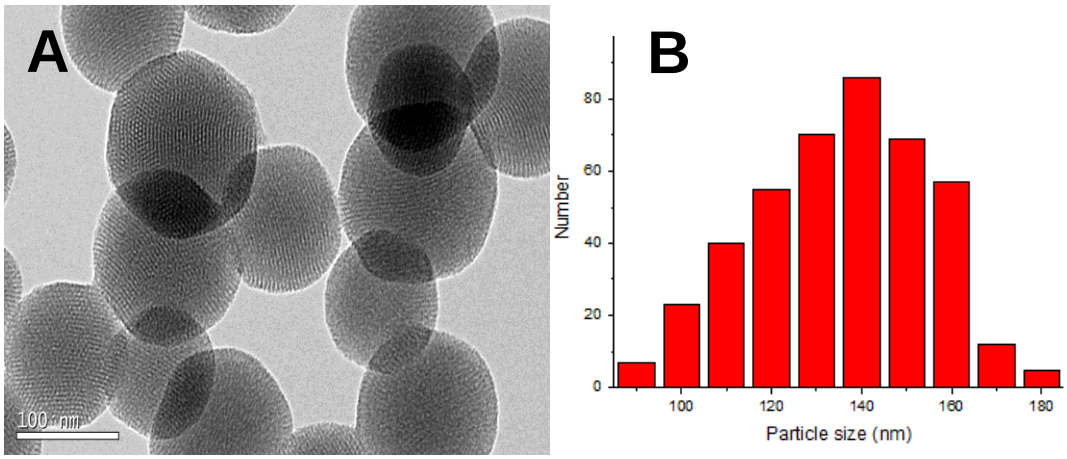


Figure 1. ***TEM (A) and size distribution (B) of MSNs***

The porous morphology of this nanomaterial was also confirmed by field-emission SEM, as illustrated in Figure 2.

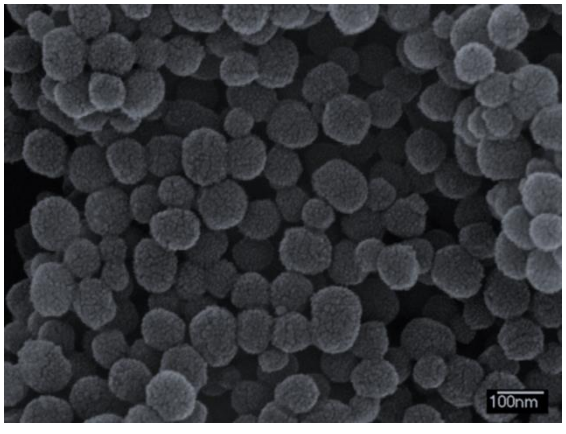


Figure 2. ***Field-emission SEM of MSNs***

The powder X-ray diffraction pattern of the both MSNs showed the mesoporous characteristic reflection peak (100) around 2.04º (Figure 3).

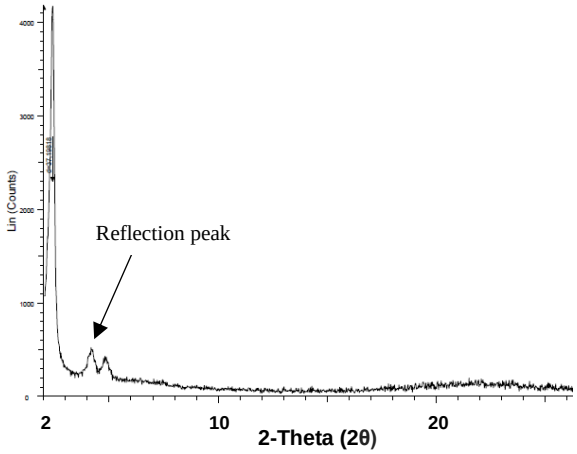


Figure 3. X-ray diffraction pattern of MSNs.

NP pore morphology was determined by nitrogen adsorption/desorption surface analysis (BET isotherms and BJH pore size distributions). Figure 4A illustrates the corresponding nitrogen adsorption/desorption isotherms and the pore size distributions for the NPs. Both MSNs and APTES-MSNs showed type IV isotherms typical of mesoporous supports. The absence of hysteresis loops suggested that all NP pores are highly accessible. On the other hand, the BET specific surface area and the average pore size (Figure 4B) were estimated as 1037 m²/g and 2.5 nm, respectively.

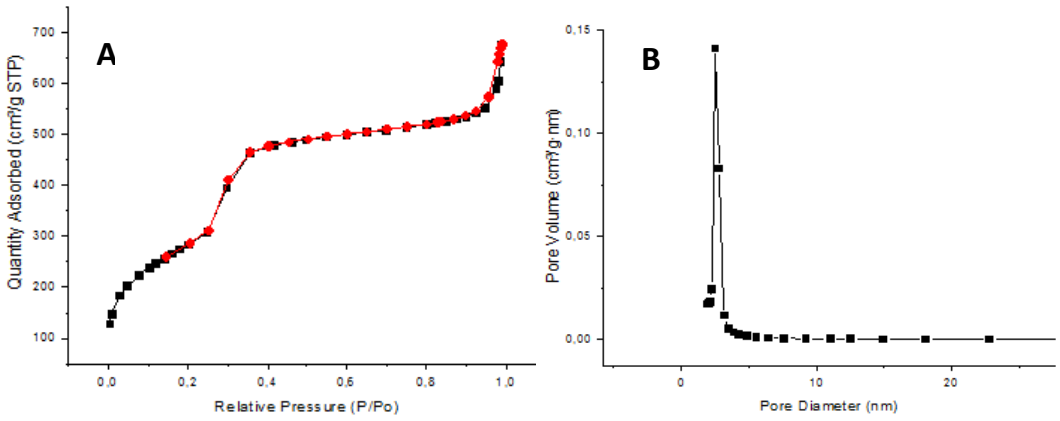


Figure 4. A) Nitrogen adsorption (black)/desorption (red) isotherms and B) pore size distribution of MSNs.

3.1.2 APTES-MSN

APTES-MSNs were obtained, as previously reported (Yokoi et al., 2012) by suspending 0.5 g MSNs in 10 mL of ethanol and by addition of the APTES solution (100 mg/mL) in a ratio of 1mL/100 mg of MSNs. The final mixture was stirred

overnight at room temperature. APTES-MSNs were collected by filtration and finally dried after two times ethanol washings.

The APTES-MSN was characterized by FT-IR (Figure 5). The strong absorbance at 1100 cm^{-1} is attributed to the Si-O-Si stretch of silica, the absorbance at 1650 and 3500 cm^{-1} is assigned to the surface hydroxyl groups of MSN.

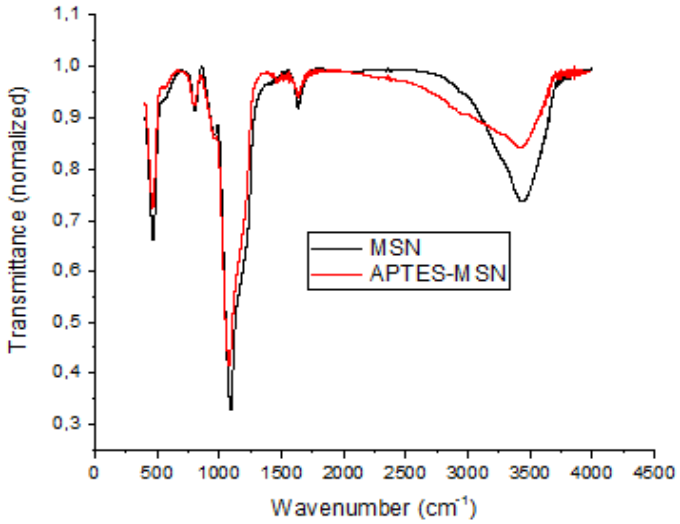


Figure 5. FT-IR analysis of MSNs and APTES-MSNs.

This modified NPs were also characterized by FT-IR (Figure 5, red line), but the amount of new chemical groups at the surface of the nanomaterials was not enough to provide evidence on the modification by FT-IR analysis.

Modification of MSNs with APTES was then confirmed by elemental analysis. It can be observed that the amount of C and N in APTES-MSN is higher compared with MSN. The average content of primary amino groups was estimated as 2.6 ± 0.2 mmol NH_2/g APTES-MSNs. 6.7 ± 0.3 mmol C/g APTES-MSNs.

Table 1. Elemental analysis of MSN and APTES-MSN

Sample	%C	%H	%N
MSN	1.04 ± 0.35	0.95 (<LOQ)	0.08 (<LOQ)
APTES-MSN	8.07 ± 0.35	2.70 (<LOQ)	3.64 ± 0.30

DSC (Figure 6) proved the thermoanalytic differences between MSN and APTES-MSN. Two distinct *Tg* values, associated with two heat capacity changes in the samples, were observed by analysing the NPs, the MSN varying from 75 to 80 °C and the APTES-MSN from 95 to 100°C. Thus, the presence of amino propyl silane groups in the NPs increased significantly the glass transition.

Enthalpy changes (ΔH) were determined by integrating the peak areas of the endotherm, being the area of the APTES-MSN significantly higher that of MSN.

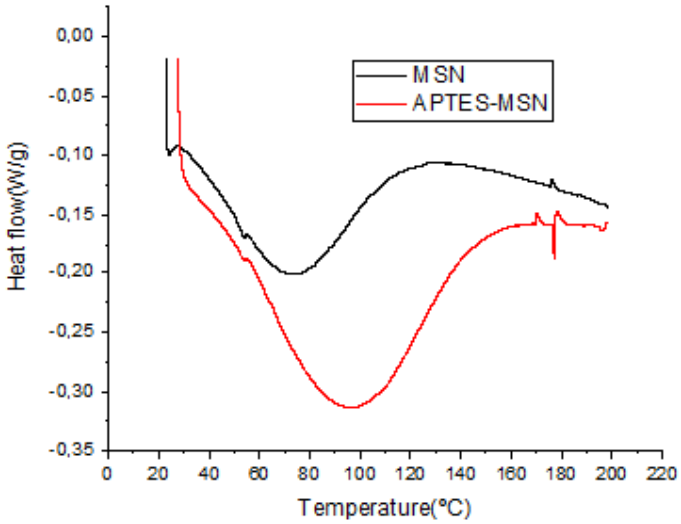


Figure 6. DSC profile of MSN and APTES-MSN.

Other experiment to prove the effective functionalization of NPs is the determination of the zeta-potential of both MSNs and APTES-MSNs (Figure 7) in the range of pH from 12 to 1 in a suspension of 1 mg of NPs /mL H_2O. Figure 7 shows that the zeta-potential of MSN, that is rich in -OH groups, was still stable at -30 mV from pH 12 to pH 8.0 and after that it linearly decreased until -25.6 mV at pH 1. This behavior is related to the protonation of the negative charge on the surface of MSNs starting at pH 7.0. (Hair Michael l. and Hert wl., 1970; Han Wu Si et al., 2013). When MSNs were functionalized by APTES, the zeta-potential determined at pH 12 (-36 mV) resulted more negative than that observed for MSNs (-30 mV). When the pH decreased even the zeta-potential of the functionalized MSNs decreased to -29 mV at pH 10, a value corresponding to the pKa of primary amino groups, and then linearly became less negative (-20 mV) of that of MSN (-25.6 mV) at very acidic pH values (under pH 4). This result confirms the structural modification of the MSN surface due to the presence of positively charged amino groups.

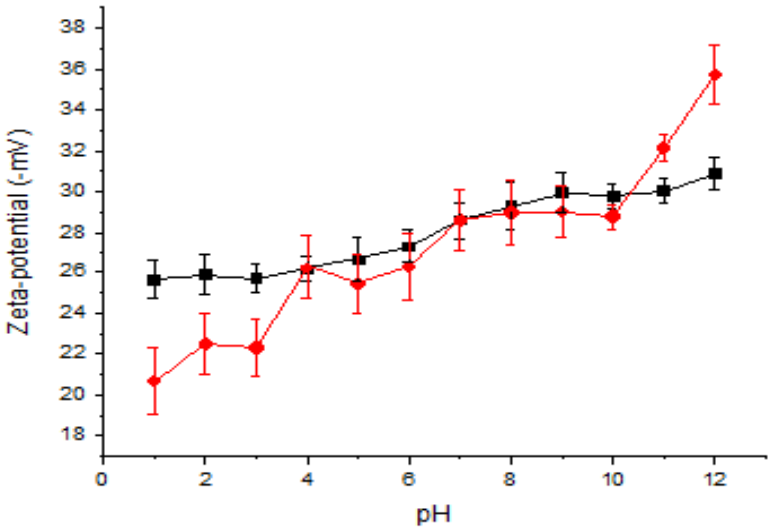


Figure 7*. Zeta-potential of MSNs (black line) and APTES-MSNs (red line) at different pH values*

This result indicates that at pH 12 both NPs showed the highest degree of dispersibility. In fact, it is well known that solutions with higher negative zeta-potential are more stable, as a consequence of the increase of repulsion among the particles that reduces their aggregation, and this effect allows obtaining a good distribution of NPs in the FFS.

3.2 Preparation and characterization of BVPC FFS and derived films

3.2.1 Characterization of BVPC FFS

In order to verify the stability of BVPC FFS, its zeta-potential was determined at different pH values (from pH 12 to pH 1). Figure 8 shows that the negative zeta-potential linearly changed from -34 mV to +2 mV with a value of 0 mV observed at pH 3. It is well known that the properties of the derived materials are associated with the homogenous distribution of the FFS components and their ability to do not aggregate before the evaporation of the solvent. This property can be correlated to the results of zeta-potential and can be useful in choosing the appropriate pH to prepare the film (Sabbah et al., 2016).

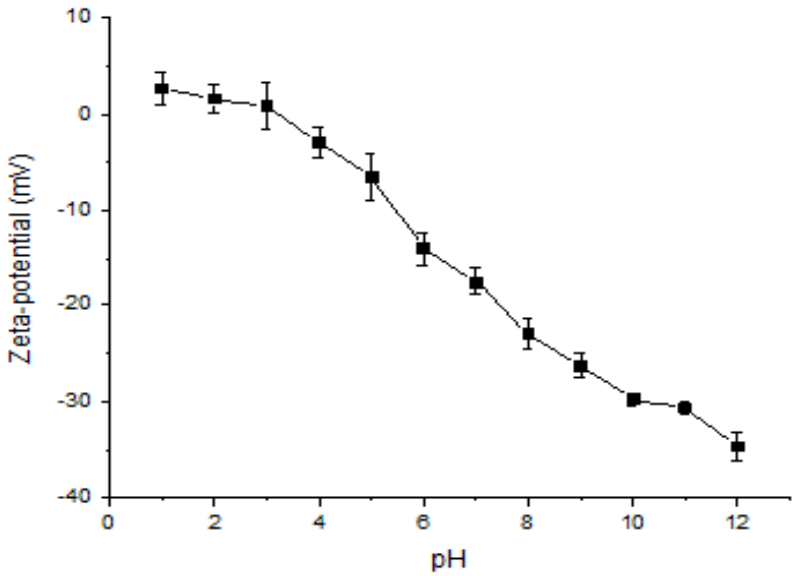


Figure 8*. Zeta-potential of BVPC FFS at different pH values*

In fact, a solution could be considered quite stable when zeta-potential is higher than ± 10 mV and very stable when it is higher than ± 30 (Suthersan et al., 2017). Therefore, in order to check the best FFS casting conditions, we prepared FFSs at pH 7, 10 and 12, and casted them to obtain handleable films and to measure their mechanical properties.

Table 2 reports that the films obtained by casting FFS at pH 12 showed better mechanical properties with respect to the films obtained at lower pHs, probably due to the higher repulsion among the protein molecules. However, pH changes did not

affect film thickness. Therefore, we prepared BVPC films at pH 12 for further experiments.

Table 2. ***Thickness and mechanical properties of BVPC films obtained at different pH values***

Film	Film TS (MPa)	Film EB (%)	Thickness (µm)
pH 7	0.63 ± 0.08	63.5 ±7.04	97 ± 5
pH 10	0.74 ± 0.07	72.4 ± 4.16	95 ± 6
pH 12	1.22 ± 0.12 [a]	114.74 ± 9.87 [a]	92 ± 2

[a] statistically significant, $p<0.01$

3.2.2 Effect of the addition of different amount of NPs to BVPC FFS and derived films

3.2.2.1 Optimization of NPs/BVPC ratio

In order to study the effect of NPs on both BVPC FFS stability and mechanical properties of the derived films, different amounts of either MSNs or APTES-MSNs (0-12% w/w with respect to protein) were added to BVPC FFS. Before casting, an aliquot of each FFS was analysed for zeta-potential and Z-average size (Table 3), while the obtained films were analysed for their mechanical properties (Figure 9). The data reported in Table 3 show that the FFS negative zeta-potential values linearly increased by adding NP amounts from 0 to 3%, then decreasing remaining almost stable between 6-12% NPs. Furthermore, a slight Z-average particle size increase was observed increasing NP concentration from 0 to 3% probably due to a homogeneous distribution of the NPs into BVPC FFS. Further increase of NP amounts determined large molecule aggregation and not detectable (n.d.) data probably due to nucleation as confirmed by the observed increase in polydispersity index (PDI).

3.2.2.2 Mechanical properties of NPs-BVPC films

Therefore, these results demonstrate that 3% was the maximum amount of NPs that can be added to BVPC FFS without its destabilization.
As far as the mechanical properties of the NP-containing films, Figure 9 shows that TS (panel A) and EB (panel B) of BVPC films increased when 3% of either MSN or APTES-MSN**,** were added to the FFS, whereas a decrease of both parameters were observed at 6 and 12% NPs. Moreover, the data reported in Table 4 indicate that NP addition always increased film thickness.

Table 3. ***Zeta-potential and Z-average of BVPC FFS prepared at pH 12 in the presence of different amounts of NPs.***

%	NP	FFS zeta-potential (-mV)	FFS PDI	FFS Z-average (d.nm)
Control	None	35.74 ± 0.78	0.47 ± 0.13	243.92 ± 3.28
1	MSN APTES-MSN	34.74 ± 0.56 36.33 ± 0.64	0.47 ± 0.09 0.49 ± 0.08	249.97 ± 2.45 248.92 ± 3.28
2	MSN APTES-MSN	36.74 ± 0.67 37.33 ± 0.42	0.49 ± 0.08 0.48 ± 0.13	273.42 ± 1.46 277.63 ± 3.28
3	MSN APTES-MSN	38.43 ± 0.59 39.72 ± 0.69	0.49 ± 0.08 0.51 ± 0.09	281.90 ± 2.87 280.76 ± 2.77
4	MSN APTES-MSN	35.19 ± 0.52 36.19 ± 0.63	0.65 ± 0.13 0.66 ± 0.11	n.d.
6	MSN APTES-MSN	33.96 ± 0.59 33.44 ± 0.49	0.69 ± 0.16 0.67 ± 0.17	n.d.
12	MSN APTES-MSN	32.91 ± 0.63 32.21 ± 0.61	0.78 ± 0.16 0.83 ± 0.12	n.d.

Table 4. ***BVPC film thickness determination at different NP concentrations***

%	NP	Thickness (μm)
Control	None	92 ± 2
3	MSN APTES-MSN	115 ± 3 114 ± 2
6	MSN APTES-MSN	107 ± 2 109 ± 2
12	MSN APTES-MSN	104 ± 2 103 ± 3

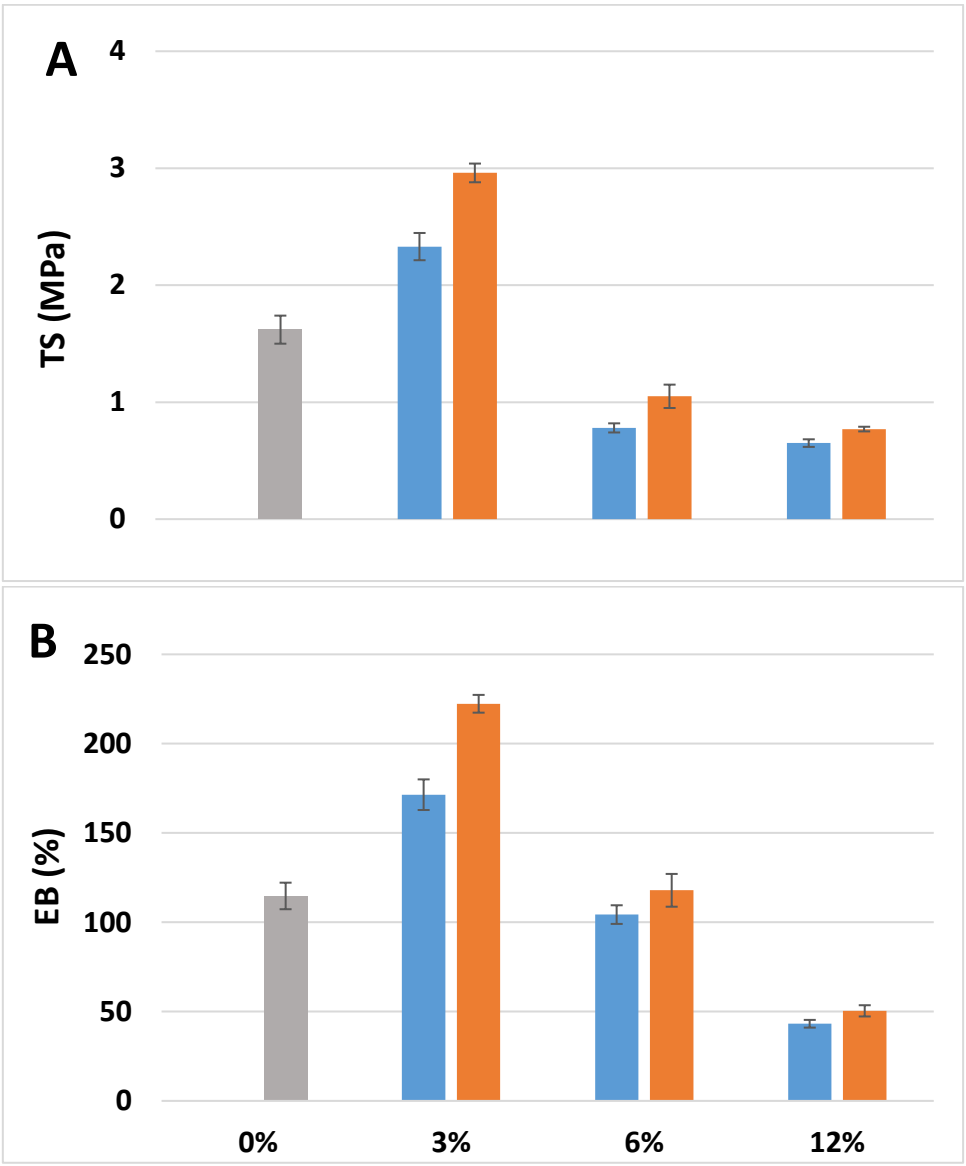


Figure 9. ***TS (A) and EB (B) of BVPC films prepared at pH 12 in the presence of different amounts of either MSN (blue bars) or APTES-MSN (orange bars) or in the absence of NPs (grey bar)***

3.3 Effect of mTGase-catalyzed protein crosslinking on the physicochemical properties of nano-reinforced BVPC films

In order to test the effect of mTGase on the nano-reinforced films, BVPC FFS was brought to pH 8, left under stirring for 15 min and finally incubated in the presence of mTGase (20 U/mg) at 25ºC for one hour. At the end of incubation, the FFS pH was adjusted to pH 12, glycerol was added (42 mM; 50% w/w protein) and the FFS stirred for 15 min before casting. Control samples were run without enzyme addition.

In order to assess the ability of NPs to influence the characteristics of the mTGase-crosslinked BVPC films, both MSN and APTES-MSN were added either before or

after BVPC incubation with the enzyme. All the obtained films had a similar homogeneous yellowish color (Figure 10).

Figure 10. ***Typical BVPC film containing NPs and mTGase***

3.3.1 Mechanical properties

Figure 11 shows that when BVPC was incubated with mTGase the mechanical properties of the obtained films did not significantly change. Conversely, BVPC treatment with mTGase effectively counteracted the observed positive effect of 3% NPs both on film TS and EB. In particular, our results indicate a more pronounced influence of mTGase when NPs were added after the enzymatic reaction occurred, i.e. when BV proteins were previously crosslinked.
This result can be explained by taking into account that the mechanical properties of the biomaterials are largely associated with distribution and density of intermolecular and intramolecular interactions of the biopolymers determining the film network.
Finally, Table 5 shows that mTGase treatment of BVPC did not seem to influence film thickness in the absence of NPs, whereas NP containing films showed a greater thickness when BV proteins were not crosslinked by the enzyme.

Table 5. ***Film thickness***

BVPC films	Thickness (μm)	
	-mTGase	+mTGase
Control	92 ± 2	89 ± 2
MSN	115 ± 3	101 ± 4
APTES-MSN	114 ± 2	103 ± 2

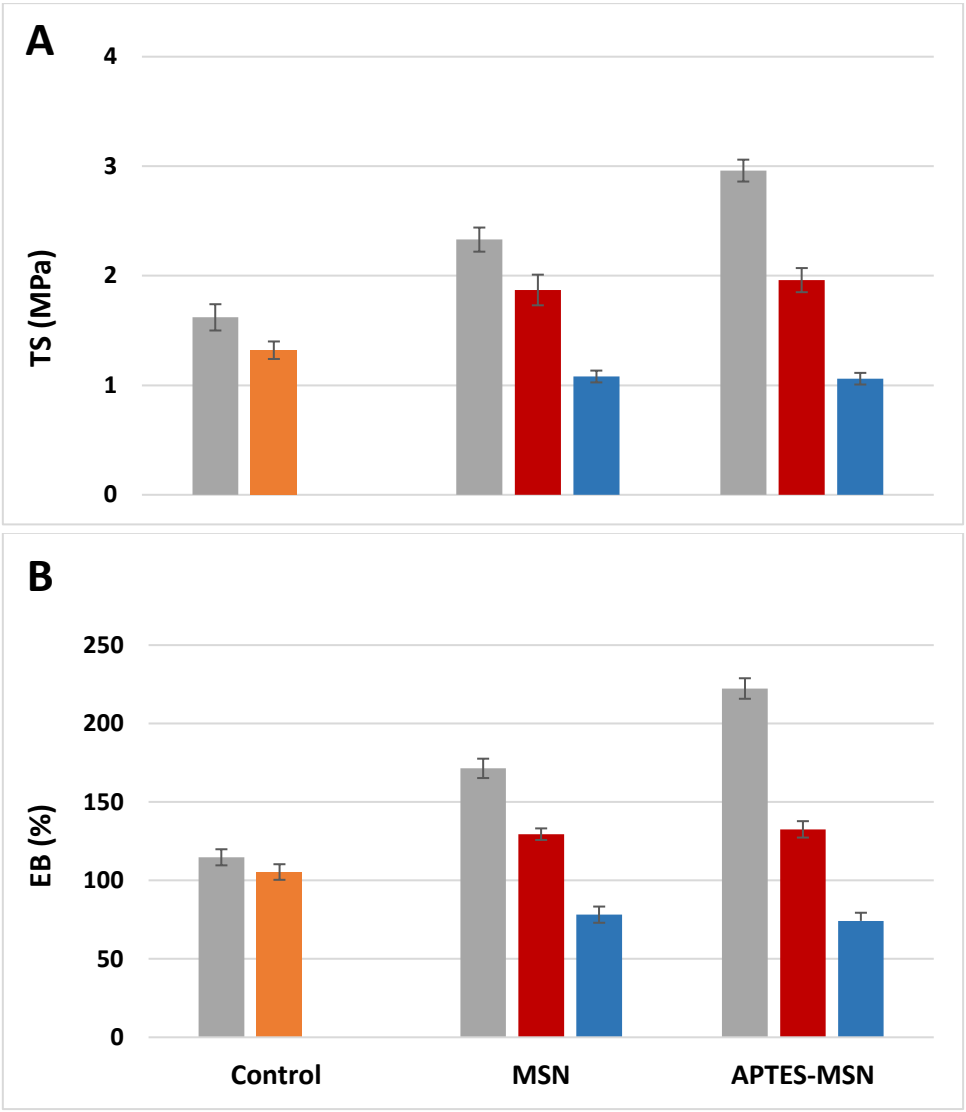


Figure 11. ***Effect of mTGase and NPs on TS (panel A) and EB (panel B) of BVPC films prepared at pH 12. BVPC was treated with mTGase and NPs were added to the incubation mixture either before (red bars) or after (blue bars) incubation. Control samples without mTGase (grey bars) and with mTGase but without NPs (orange bar) were run simultaneously.***

3.3.2 Barrier properties

In Table 6 the barrier properties of BVPC edible films in absence or presence of NPs and treated or not with mTGase are reported. The data indicate that the addition of NPs, mostly APTES-MSN, has a positive barrier effect on BVPC films toward both gases and water vapor. This effect was even higher when the films were prepared by using mTGase-pretreated BVPC.

Table 6. ***Effect of mTGase pretreatment of BVPC on the permeabilities of the derived films prepared at pH 12 either in the absence or presence of NPs***

Permeability (cm^3 mm / $m^{-2}d^{-1}KPa^{-1}$)	Control		MSN		APTES-MSN	
	-TGase	+TGase	-TGase	+TGase	-TGase	+TGase
CO_2	2.36 ± 0.23	1.36± 0.18	1.12 ± 0.03	0.21 ± 0.02	0.32 ± 0.01	0.23 ± 0.05
O_2	3.42 ± 0.71	0.20 ± 0.08	0.36 ± 0.05	0.23 ± 0.07	0.28 ± 0.02	0.21 ± 0.03
WP	2.12 ± 0.37	0.13 ± 0.02	0.09 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.02 ± 0.01

3.3.3 Morphological properties

NP containing films made with mTGase-treated and -untreated BVPC were characterized after metallization through SEM at 5 kV. Figures 12-15 show the SEM images of the surface (Figure 12) and of the cross section (Figures 13-15) of the different samples.

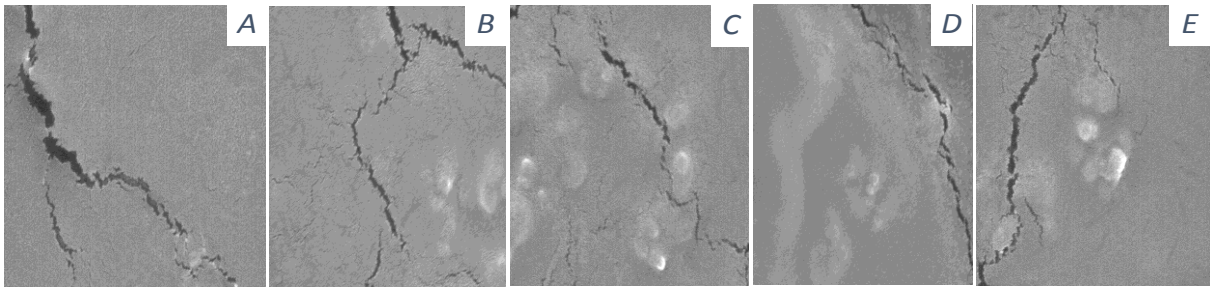


Figure 12. ***SEM images (x 100) of surfaces of films made with untreated BVPC (A), MSN-containing films made with either untreated BVPC (B) or mTGase-treated BVPC (C)***, ***APTES-MSN-containing films made with either untreated BVPC (D) or mTGase-treated BVPC (E)***.

All the images of mTGase-treated BVPC films containing NPs clearly show a more compact microstructure of the film network, with evident continuous zones, in comparison with the control samples, the zoomed image of the sections indicating the effect of the enzyme in a homogenously inclusion of NPs into the film.

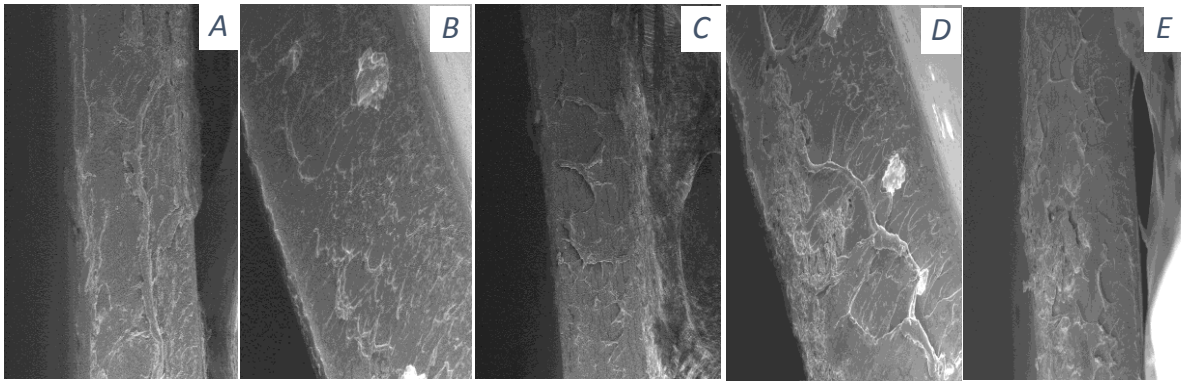


Figure 13. ***SEM images (x 700) of cross sections of films made with untreated BVPC (A), MSN-containing films made with untreated BVPC (B) or mTGase-treated BVPC (C)***, ***APTES-MSN containing films made with either untreated BVPC (D) or mTGase-treated BVPC (E)***.

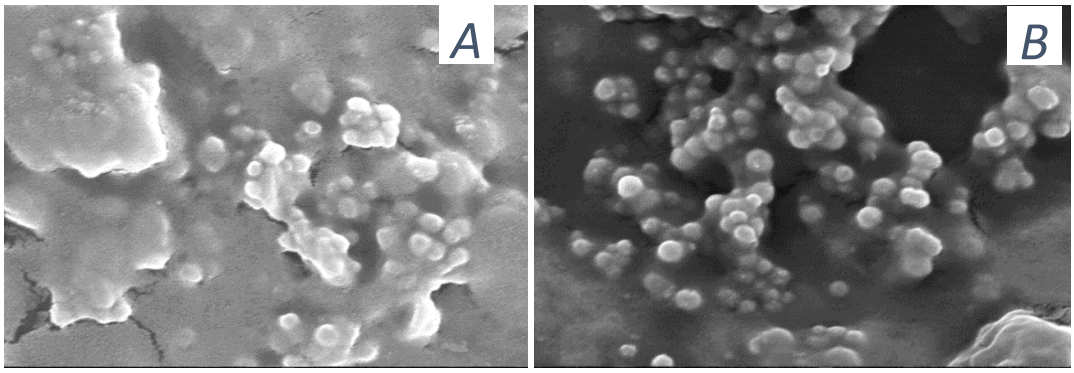


Figure 14. ***SEM images (x 3,000) of cross sections of MSN-containing films made with untreated BVPC (A) or mTGase-treated BVPC (B)***

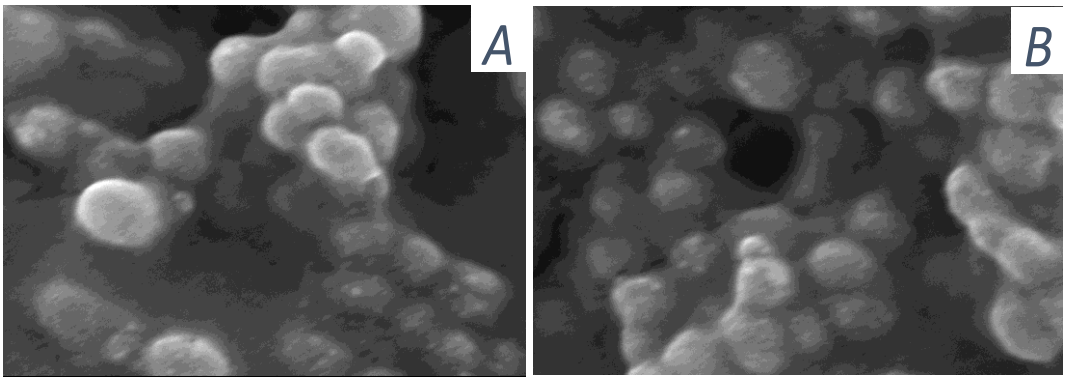


Figure 15. ***SEM images (x 10,000) of cross sections of APTES-MSN-containing films made with untreated BVPC (A) or mTGase-treated BVPC (B).***

Regarding to AFM experiments, the initial analyses of BVPC edible films were performed using a tripod scanner to provide large areas for a general view of the film surface. Although the first examinations were performed in the noncontact scanning mode, contact mode imaging was attempted to reduce noise from mechanical

vibration of the microscope. Thus, we determined that high quality images could be obtained when the contact mode was employed using a tripod scanner.
A roughness value (Rq) of 61.42 nm was calculated for BVPC films (control samples), whereas Rq values of 31.35 nm and 25.33 nm were determined for MSN-containing films and APTES-MSN-containing films, respectively, mTGase BVPC treatment having no effects (Figure 16).

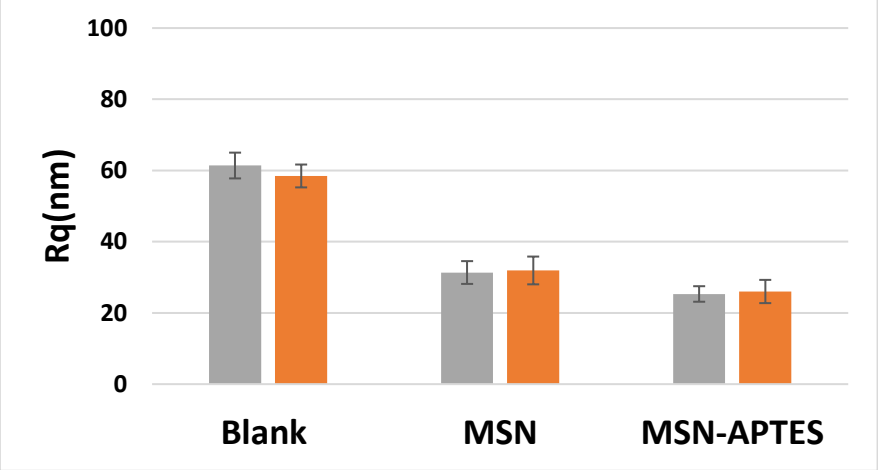


Figure 16. ***Rq values of MSN-and APTES-MSN-containing films made with either untreated (grey bars) or mTGase-treated BVPC (orange bars).***

The roughness decrease is probably the result of the higher film homogeneity at the nanoscale determined by the NP inclusion into the protein network which could be responsible for an increasing smoothness of the matrices obtained.

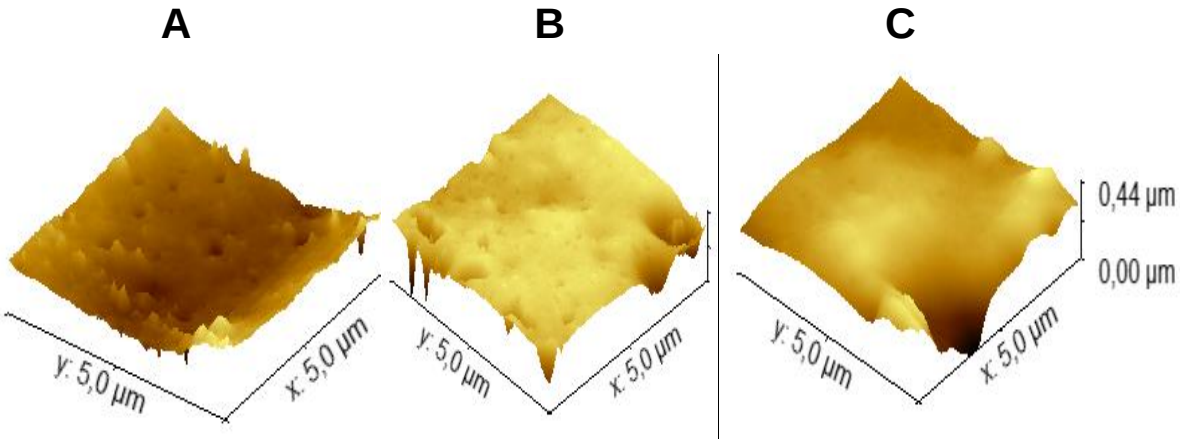


Figure 17. ***Typical AFM images of BVPC films prepared both in the absence (A) and presence of either MSNs (B) or APTES-MSNs (C).***

3.3.4 Thermoanalytic properties

DSC analyses of all the prepared films were also carried out. Film weight was determined to calculate the change in enthalpy expressed as joules per gram of the dry matter. Glass transition temperature (*Tg)* was obtained from the leading edge of

the melting of phase transition, whereas melting temperature (*Tm)* from the lowest point of melting of the phase transition on the temperature axis (Jagannath et al., 2006). The results reported in Figure 18 showed the behaviour of the different BVPC films. Two distinct *Tg* values, associated with two heat capacity changes in the samples, were observed by analysing all types of film, the first varying from 55 to 60 °C and the second one from 90 to 105°C. The presence of MSNs decreased the second glass transition of films made with both untreated and mTGase-treated BVPC from 105°C to 95°C and 85°C, respectively, creating also an inverse curve which is more pronounced in the mTGase-treated BVPC films. A similar effect was observed by analysing the APTES-MSN-containing BVPC films, with the exception of the opposite result obtained with the films prepared with mTGase-treated BVPC which clearly show an increase of the second glass transition (110°C).
Enthalpy changes (ΔH) were determined by integrating the peak areas of the endotherm. The higher value of ΔH indicated that a more extensive network had formed (Jagannath et al., 1998).

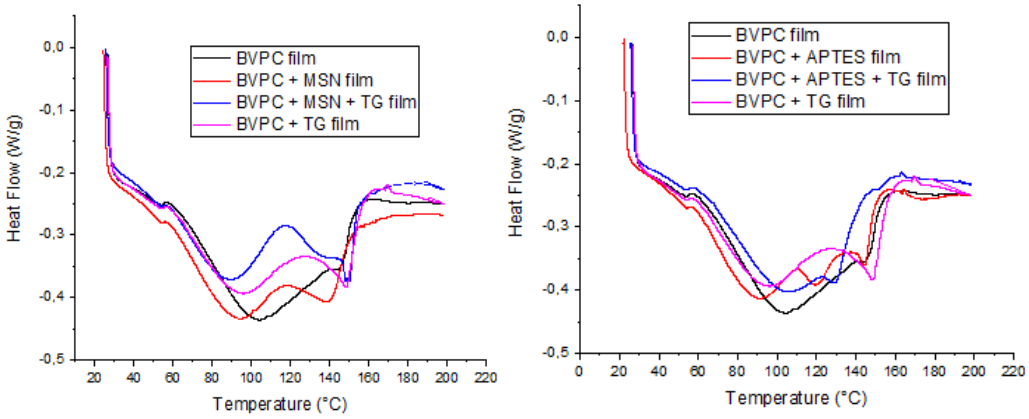


Figure 18. ***DSC profile of MSN- (left) and APTES-MSN (right) -containing films made with either untreated or mTGase-treated BVPC***

A thermogravimetric analysis of BVPC films was also carried out in order to study the influence of NPs and mTGase on the film thermal stability. Results reported in Figure 19 indicate a similar weight loss vs. temperature in all curves obtained by analysing the films containing or not both NPs and mTGase.

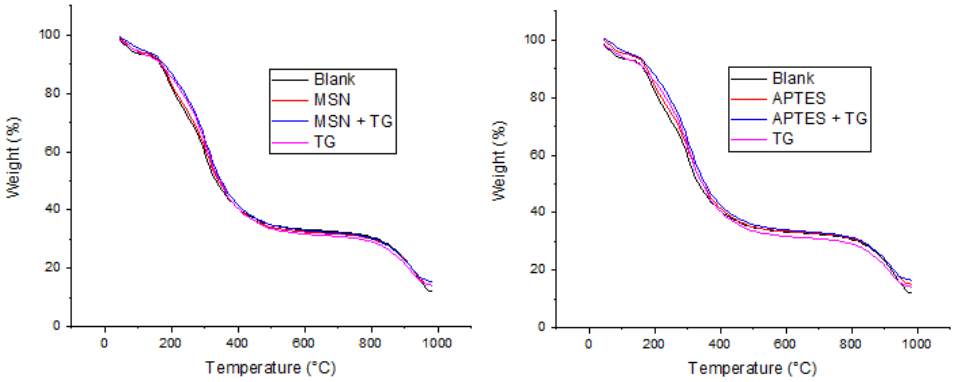


Figure 19. ***Thermogravimetric profile of NPs-containing films made with either untreated or mTGase-treated BVPC***

3.3.5 FT-IR characterization

The FT-IR spectra of BVPC films prepared in the absence and presence of either MSNs or APTES-MSNs show the contribution of different functional groups belonging not only to BV proteins but also to carbohydrates and others small molecular weight compounds contained in BVPC (Figure 20).

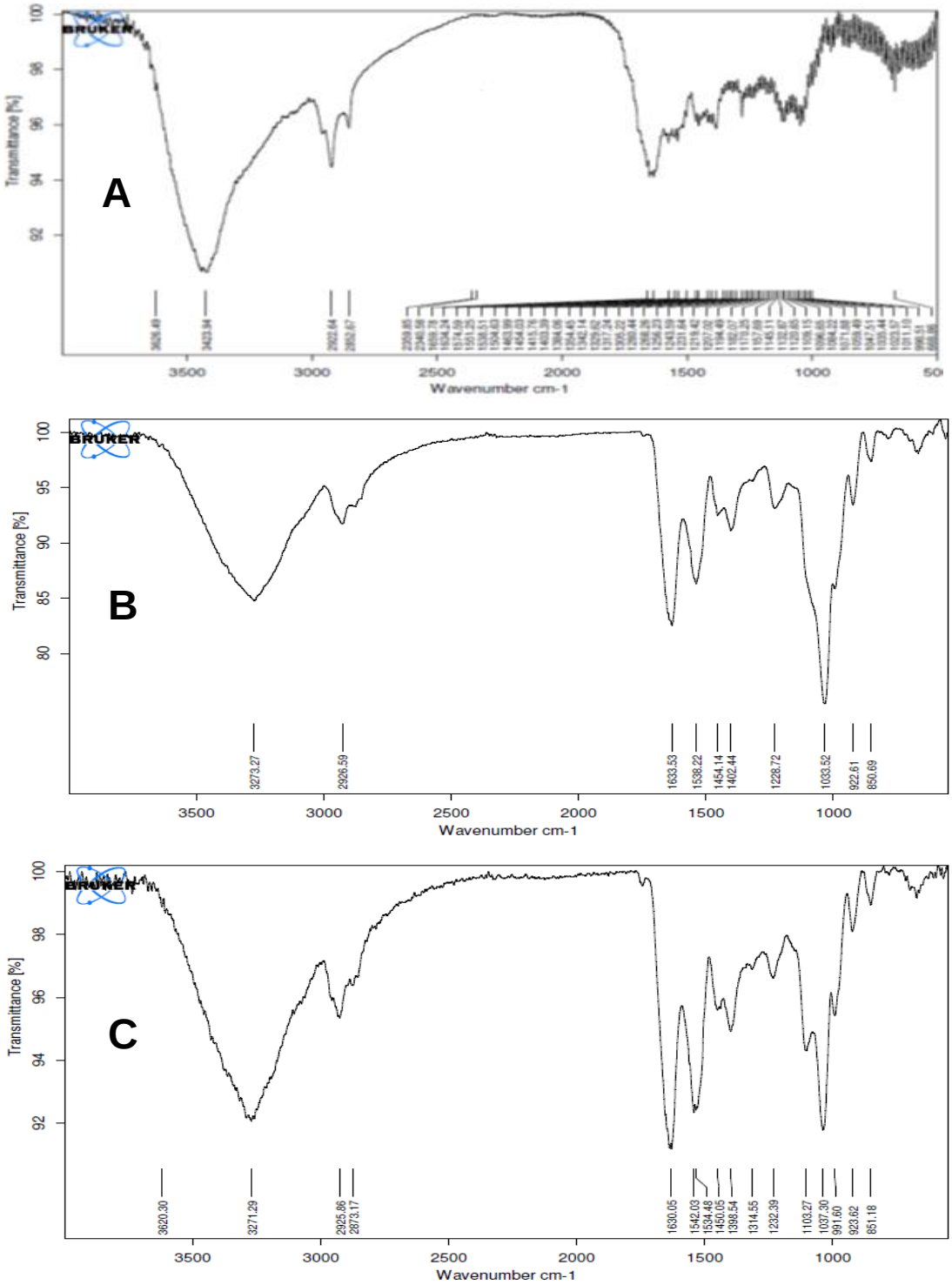


Figure 20. FT-IR spectra of BVPC films prepared in the absence (A) and presence of either MSNs (B) or APTES-MSNs (C)

Panels A, B and C of Figure 20 indicate that there were no differences in the profile shape of transmittance, even though some differences in the peaks intensities were observed.

The broad band ranging between 3100 and 3500 cm^{-1} corresponds to stretching of O–H and N–H bonds, respectively (Martins et al., 2010), whereas the peak marked at 2928 cm^{-1} is attributed to C–H stretching vibration.

Other peaks were observed in the region of 1633 cm^{-1}, corresponding to the symmetrical stretching of the amide group. The peak at around 1500 cm^{-1} is attributed to amide II band, arising from bending vibration of N-H groups and stretching vibrations of C–N groups. The bands in the region between 1100 and 900 cm^{-1} are considered characteristic of saccharides and are attributed to the stretching of C–C and C–O bonds, with some contribution from C–H bonds.

3.3.6 Biological properties

In order to evaluate the possibility to prepare an “active” edible film by using it as carrier of bioactive molecules we have added the antimicrobial agent nisin in the BVPC FFS and tested the antimicrobial activity of both FFS and derived edible film by the diffusion agar test using the *Micrococcus luteus* as microorganism. Antimicrobial activity of nisin was measured in International Units (IU), and the activity approximately of 1 µg of pure nisin was of 40 IU (Rossi-Marquez, 2009). Thus, to evaluate the antimicrobial properties we added nisin at a concentration of 1 IU/mL BVPC FFS. Antibacterial effect of BVPC edible film against *Micrococcus luteus* was expressed in terms of zone of inhibition.

3.3.6.1 Antimicrobial activity of BVPC FFS

Once prepared the agar with *Micrococcus luteus*, a hole with a diameter of 6 mm is punched aseptically with a sterile cork borer, and 40 µL of FFS are introduced into the well. Then, agar plates are incubated at 30°C for 24 hours. The inhibition halo diameter was measured with a calliper by holding the ruler of calliper on the underside of the Petri dishes and making a direct reading in millimetres of multiple zones to obtain an average size.

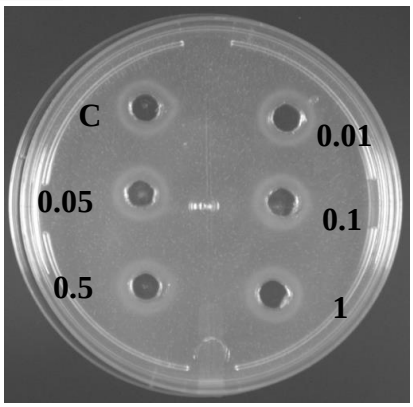


Figure 21. ***Antimicrobial activity of BVPC FFS (10 mg/ml) in the absence (c) or presence of different amounts of nisin (from 0 to 1 IU/mL).***

BVPC FFS was shown to possess antimicrobial activity (Figure 21) also in the absence of nisin added at different amounts. Moreover, the presence of either 3% (w/w) NPs or 20 U/g mTGase, as well as of both additives, were shown to do not influence the antimicrobial activity exhibited by BVPC (data not shown). In fact, the halo diameter was observed to minimally grow following addition of different nisin amounts, being not appreciated considerable increases of the zones of inhibition.

3.3.6.2 Antimicrobial activity of BVPC films

Hold the ruler or caliper on the underside of the Petri dish and make a direct reading in millimeters. Include the size of the antimicrobial sample in the measurement. Take readings of multiple zones to obtain an average size. Film samples were cut, using a sharp razor blade, into 10-11 mm diameter pieces, placed on the agar with *Micrococcus luteus,* and finally incubated at 30°C for 24 hours. Then, the measure of the inhibition halo diameter with a caliper to the nearest 0.01 mm was carried out.

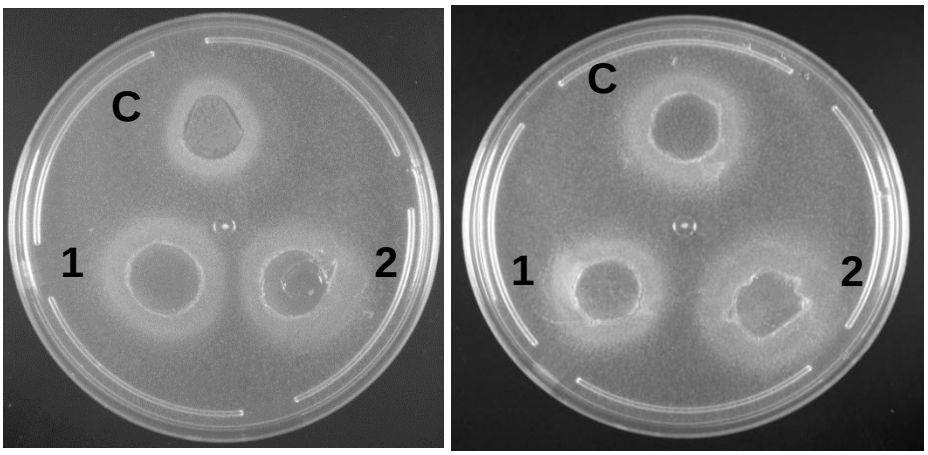


Figure 22. Antimicrobial activity of nisin (a, 1 IU/mL; b, 2 IU/mL) containing BVPC films prepared in the presence of MSNs (A) and APTES-MSNs (B). Control samples were carried out with BVPC film prepared without nisin.

Figure 22 shows that also BVPC film exhibits an antimicrobial activity in the absence of nisin and that nisin addition increases the inhibition zones from 10 to 16 mm (by adding 1 U nisin/ml) and from 16 to 18 mm (by adding 2 U nisin/ml). Equivalent results for APTES-MSN, being a little bit higher than MSN. Similar results were obtained also with BVPC films in the absence and presence of 20 U/g mTGase.

Table 7. Inhibition zone of BVPC films

BVPC film	Inhibition zone (mm)	
	MSN	APTES-MSN
Control	10 ± 2	12 ± 1
1	16 ± 1	17 ± 1
2	18 ± 1	19 ± 1

3.3.6.3 Antifungal activity of BVPC films

To study the ability of edible films to stop the growth of fungi, we have used a standard methodology, described in the Materials and Methods, using as inoculum a mixture of *Aspergillus niger*, *Penicillium pinophilum, Chaetomium globosum*, or *Aureobasidium pullulans.*

BVPC films, prepared in the absence or presence of NPs and mTGase and containing or not 1 IU nisin/mL, as well as a filter paper used as control, were cut by using a sharp razor blade into 20-25 mm diameter pieces and placed on the agar. Sample surfaces were inoculated with the spore mixture by spraying the suspension by a sterilized atomizer with 110 KPa (16 psi) of air pressure so that the entire surface was moistened. All the samples were finally incubated at 30ºC and not less than 85% relative humidity for 10 days. Figure 23 clearly shows a high growth of fungi on the filter paper after the third day whereas the growth of fungi on the BVPC films started after 5 days probably due to the inherent film antifungal activity. Such activity was significantly improved when nisin was present into the films. Same results were obtained by using BVPC films containing NPs and mTGase both alone and combined.

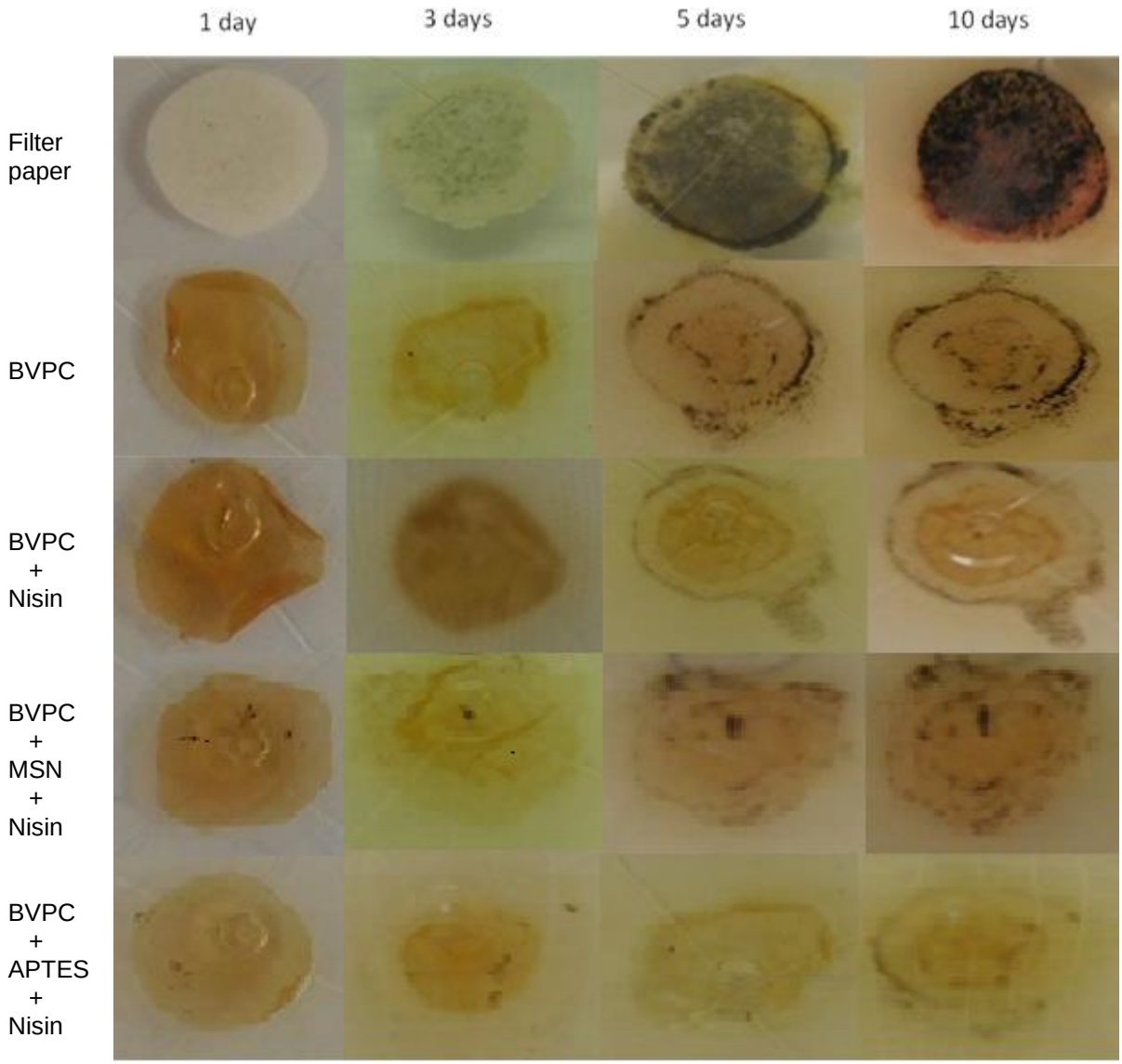


Figure 23. Antifungal activity of both filter paper (first lane) and BVPC films, either alone (second lane) or containing 1 IU nisin/mL in the absence (third lane) or presence of MSNs (fourth lane) or APTES-MSNs (fifth lane). Biodegradation was observed at different days (1, 3, 5 and 10 days, respectively) after inoculation. Further experimental details are given in the text.

4.CONCLUSIONS

A variety of BVPC edible films -i.e. reticulated by transglutaminase, containing two different NPs (MSNs and APTES-MSNs) and/or the bioactive oligopeptide nisin, as well as all the possible combinations of these experimental conditions- were produced and characterized for their physicochemical, morphological and biological properties. MSNs, as well as functionalized MSN (APTES-MSN), were preliminary synthesized to obtain NPs with low polydispersity index and homogeneous distribution of pores.

The addition of NPs to BVPC FFS had a positive effect on both mechanical (tensile strength and elongation at break) and barrier properties (CO_2, O_2 and water vapor permeability) of the derived cast films. Moreover, the presence of amino groups instead of hydroxyl groups on the surface of the NPs showed a significant improvement of film properties, probably due to the interaction of the APTES positive charges with the negative ones of BV proteins that would allow a more homogeneous distribution of NPs during FFS casting. Moreover, the introduction of mTGase-catalyzed crosslinks into the protein matrix of the films gave rise to a more rigid film structure with the result to counteract the improvement of the mechanical properties promoted by NPs but, decreasing the biomaterial free volume, the enzyme treatment further increased film barrier properties to both gases and water vapour. Finally, the biological experiments carried out with all types of biomaterial showed an intrinsic antimicrobial and antifungal activity of the BVPC films that was not affected by neither the presence of NPs nor by the BV protein enzymatic reticulation. Conversely, the addition of nisin, a well-known antimicrobial and antifungal oligopeptide, determined a dose-dependent increase of both biological activities of BVPC films, thus indicating that nisin kept its effects also when it was incorporated into the film matrix.

In conclusion, the use as filler of silica NPs to obtain nanocomposite BV protein-based films has proved to be an effective strategy to improve the mechanical and barrier properties of such edible films, whereas mTGase was shown to be a useful tool to further increase only film barrier properties. Furthermore, BVPC films showed an intrinsic antimicrobial and antifungal activity that increased by nisin addition. These findings, therefore, suggest the possibility to produce different tailored BV protein films as active food coatings according to the specific requirements of product preserving.

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6. APPENDIX

1. Experience in foreign laboratories

a. Department of Analytical Chemistry, Complutense University of Madrid (Spain)
Supervisor: Prof. Reynaldo Villalonga Santana
Topic: Synthesis of NPs (MSNs and APTES-MSNs)
Date: September 2015

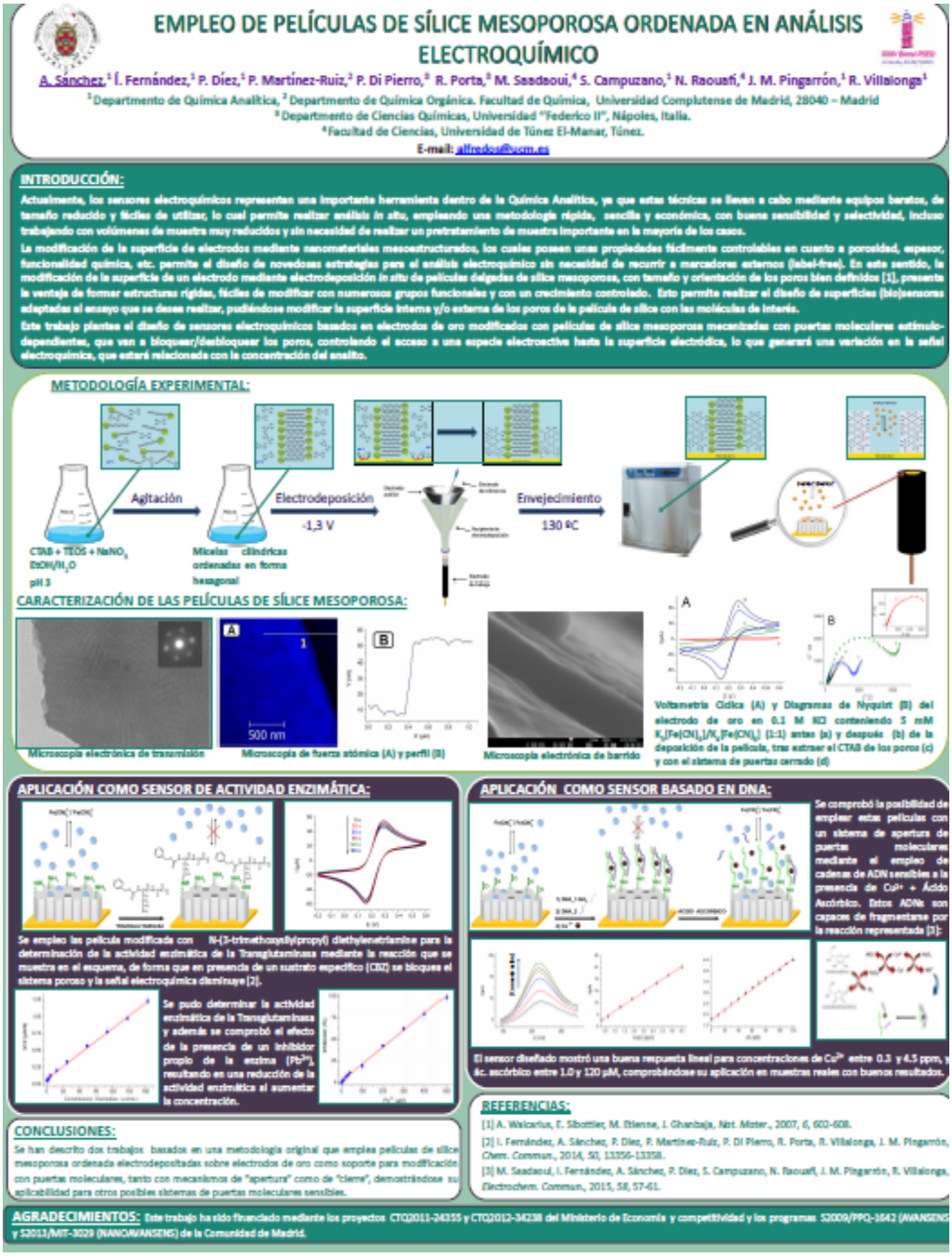
b. Department of Analytical Chemistry, Complutense University of Madrid (Spain)
Supervisor: Prof. Reynaldo Villalonga Santana
Topic: Analysis of BVPC edible films by microscopy instruments (SEM, AFM)
Date: July 2016

c. Stage in Department of Food Biotechnology, Autónoma University of Querétaro (México)
Supervisor: Prof. Carlos Regalado
Topic: Study of antimicrobial and antibacterial properties of edible films
Funding Institution: Researcher mobility funded by the fourth executive program of scientific and technological cooperation between Italy and the United States of Mexico
Date: October 2016 –March 2017

d. Department of Analytical Chemistry, Complutense University of Madrid (Spain)
Supervisor: Prof. Reynaldo Villalonga Santana
Topic: Physicochemical characterization of nanoparticles and BVPC films
Date: July 2017- September 2017

2. Contributions to scientific meetings

a. Contribution: Poster
Congress: XXXV Bienal RSEQ, University of A Coruña (Spain)
Date: 19/07/2015 - 23/07/2015



b. Contribution: Poster
Congress: Advanced training course on emerging biotechnologies for the sustainable waste management. XXXI IUPAC Chemrawn conference, Naples (Italy)
Date: 4/04/2016 - 6/04/2016

PREPARATION AND CHARACTERIZATION OF BIOPLASTICS FROM GRASS PEA PROTEINS CAST IN THE PRESENCE OF TRANSGLUTAMINASE

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giosafat@unina.it

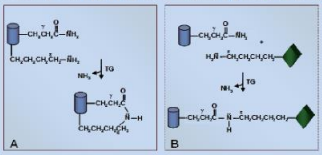
Biotecnologie Biochimiche ed Enzimologia

BIOPLASTICS were prepared from proteins from GRASS PEA (*Lathyrus sativus*) a low production cost legume cultivated specially in the past for seeds for animal and human consumption. It adapts to harsh environments, being resistant to drought and low quality of soil. Grass pea is as good source of proteins and carbohydrates, resistent to a biotic stress, and as pulse is able to fix nytrogen.

BIOPLASTICS: Bio-based eco-products with commercial and environmentally acceptability that are derived from renewable resources and have recycling capabilities and or/triggered biodegradability (*Soroudi and Jakubowicz, 2013*)

GRASS PEA seeds

TRANSGLUTAMINASE **catalyzed reactions**



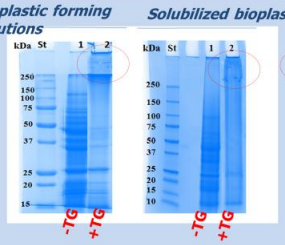
Intra-molecular (A) and Inter-molecular (B)crosslinks

TRANSGLUTAMINASE, an enzyme able to catalyze intra- and inter-molecular crosslinks between glutamines and lysines, was used as biotechnological tool (Giosafatto et al., 2014) to prepare bioplastics using casting method.

Grass pea protein-based bioplastics

Transglutaminase (microbial isoform) was able to use grass pea proteins a substrates, as demonstrated by SDS-PAGE analisys of bioplastic forming solutions (pre-casting samples) and solubilized bioplastics (post-casting samples)

Bioplastic forming solutions ***Solubilized bioplastics***

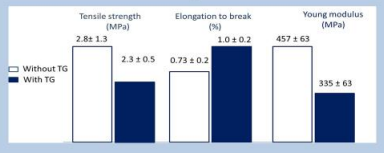


TG-mediated polymers

SDS-PAGE (12%) analisys of grass pea protein-based bioplastics

The bioplastics were characterized according to their MECHANICAL PROPERTIES. Results demonstrate that TG significantly influences Young Modulus, thus providing more elastic bioplastics compared to the ones prepared without the aim of the enzyme.

MECHANICAL PROPERTIES
of grass pea proteins-based bioplastics



CONCLUSIONS and FUTURE PERSPECTIVES

- ✓ Grass pea proteins (unlike proteins from other sources) are able to give rise to bioplastics without the need of polysaccharides.
- ✓ Microbial TG is able to modify grass pea proteins during casting
- ✓ Microbial TG improves film elasticity
- ✓ Further characterizations are needed to decide field applications of such bioplastics

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- Giosafatto *et al.* (2014) Carb. Polym. 106: 200-208.
- Soroudi & Jakubowicz I (2013) Eur Polym J 49(10):2839-2858

c. Contribution: Poster
Congress: Solid urban waste management. XXXI IUPAC Chemrawn conference, Rome (Italy)
Date: 6/04/2016 - 8/04/2016

Protein edible films reinforced with nanoparticles acting as transglutaminase substrates

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Introduction

The development of nanocomposites is increasingly employed to improve physical properties of polymer films (1). We suggest a new strategy to reinforce edible films containing proteins able to act as acyl donor substrates of transglutaminase (TGase) by using nanoparticles functionalized with amino moieties as acyl acceptors for the enzyme. The proteins used were extracted from seeds of bitter vetch *(Vicia ervilia)*, an ancient grain legume crop of the Mediterranean region **(Fig. 1)**. To improve the structural network of bitter vetch protein (BVP) films we used TGase (2), mesoporous silica nanoparticles (MSN) and MSN functionalized with aliphatic chains endowed with primary amino groups (APTES, 3-aminopropyl-triethoxysilane). Finally, the obtained BVP films were characterized for their mechanical properties.

Figure 1

Fig 2 shows the images obtained by transmission electron microscopy of the MSN-APTES size (A) and surface (B) and that of the resulting film (C).

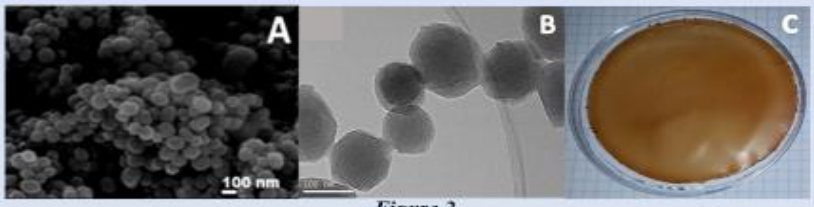

Figure 2

Table 1 shows the mechanical (tensile strength and elongation to break) properties of the films obtained in the presence of the different nanoparticles with or without enzyme.

BVP film	Tensile strength (MPa)		Elongation to break (%)	
	-TGase	+TGase	-TGase	+TGase
Without MSN	1,63 ± 0,23	1,97 ± 0,35	129,95 ± 14.7	107,21 ± 4,17
with MSN	2,13 ± 0,35	1,93 ± 0,47	181,62 ± 18,8	118,48 ± 8,17
with MSN-APTES	1,84 ± 0,37	1,38 ± 0,35	195,33 ± 13,03	115,01 ± 7,58

Table 1

Conclusions

- MSN-APTES is substrate of TGase
- The addition of MSN and MSN-APTES improve the mechanical properties of the edible film

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Acknowledgement
This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the "Fourth Executive Program of Scientific and Technological Cooperation between Italy and the United States of Mexico" ,2015.

d. Contribution: Oral presentation
Congress: Designer biology: From proteins and cells to scaffolds and materials, Vienna (Austria)
Date: 7/06/2017 - 9/06/2017

Improved physicochemical properties of bitter vetch (*Vicia ervilia*) transglutaminase crosslinked protein film by APTES-modified mesoporous silica nanoparticles

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The major limit of protein-based films in food packaging is their relatively poor mechanical and barrier properties which currently hinders their industrial use. The most frequently used strategy to improve film characteristics is to prepare composite materials which consist of a polymer matrix (continuous phase) and a filler (discontinuous phase). The advancement of nanotechnology has boosted interest to new types of composites in which the filler has at least one dimension smaller than 100 nm (nanocomposites). They exhibit generally increased barrier properties and mechanical strength, as well as improved heat resistance compared to their neat polymers and conventional composites.

In our work we suggest a new strategy to synthetize nano-reinforced biomaterials by using as polymer matrix the proteins from bitter vetch (BVP) and as filler the mesoporous silica nanoparticles (MSN) functionalized with (3-aminopropyl) triethoxysilane (APTES). To improve the structural network, the film forming solution was incubated in the presence of transglutaminase (TGase), a protein crosslinking enzyme. The obtained results showed that all the BVP films reinforced with MSN or MSN-APTES were able to markedly improve the mechanical and barrier properties to both gases and water vapor, and that TGase BVP treatment further reduced film permeability.

Acknowledgements: This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the "Fourth Executive Program of Scientific and Technological Cooperation between Italy and the U.S. of Mexico ", 2016.

e. Contribution: Poster
Congress: 59th Società Italiana di Biochimica (SIB) congress, Caserta (Italy)
Date: 20/09/2017 - 22/09/2017

Transglutaminase-crosslinked protein-based films reinforced by mesoporous silica nanoparticles

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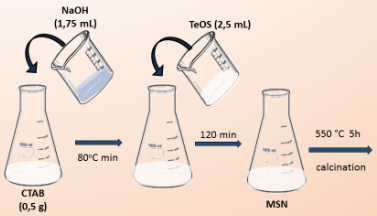
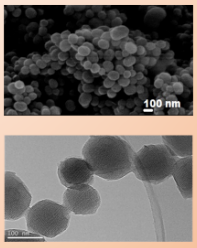
Introduction

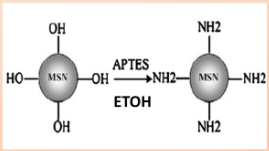
We propose a new strategy to produce nano-reinforced biomaterials by using as polymer matrix proteins extracted from bitter vetch seeds and, as filler, mesoporous silica nanoparticles (MSN) functionalized or not with (3-aminopropyl)-triethoxysilane (APTES). To improve the structural network, the nanoparticle containing film forming solution (FFS) was incubated in the presence of transglutaminase (TG, EC 2.3.2.13), a protein crosslinking enzyme, of microbial origin.

Methodology

MSN were synthesized by using tetraethoxysilane (TeOs) in the presence of cetyltrimethylammonium bromide (CTAB) as surfactant. At the end of the reaction the surfactant was take off by calcination to create the mesostructured material. The functionalized MSN (APTES-MSN) were obtained incubating MSN and APTES in ethanol (ETOH) overnight under stirring .
Films were prepared by mixing bitter vetch protein concentrate (BVPC) (10 mg/mL) with 42 mM glycerol (50% w/w protein), MSN or APTES-MSN (3% w/w protein), TG (20 U/g of protein) at pH 12, and casting FFSs at 9.7 mg/cm^2 in Petri dishes finally dried for 48 h at 25°C (45 % RH) .

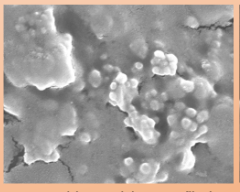




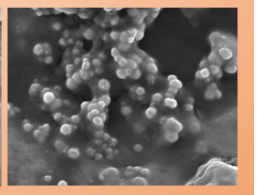


Results

All the BVPC films reinforced with nanoparticles showed improved mechanical properties, such as tensile strength (TS) and elongation at break (EB), and reduced permeabilities to gases (O_2 and CO_2) and water vapor (WV). TG addition further improved film barrier properties but counteracted the improvements of mechanical properties observed by using nanoparticles. SEM images indicated that, in the presence of the enzyme, an aggregation of both types of nanoparticles (MSN and APTES-MSN) occurred in the protein crosslinked network. This phenomenon could be responsible for counteracting the improvement of mechanical properties previously observed with films prepared without TG.



Nanoparticle-containing BVPC film in the absence of TG



Nanoparticle-containing BVPC film in the presence of TG

		Control		MSN		MSN-APTES	
		-TG	+TG	-TG	+TG	-TG	+TG
Permeability ($cm^3\ mm\ m^{-2}\ d^{-1}\ kPa^{-1}$)	CO_2	2.36 ± 0.23	1.36±0.18	1.12 ± 0.03	0.21 ± 0.02	0.32 ± 0.01	0.23 ± 0.05
	O_2	3.42 ± 0.71	0.20 ± 0.08	0.36 ± 0.05	0.23 ± 0.07	0.28 ± 0.02	0.21 ± 0.03
	WV	2.12 ± 0.37	0.13 ± 0.02	0.09 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.02 ± 0.01
TS (MPa)		1.62 ± 0,13	1.32 ±0.07	2.33 ± 0.09	1.47 ± 0.11	2.64 ± 0.09	1.45 ± 0.13
EB (%)		114.74 ± 4,43	105.35 ± 3.28	191.43 ± 3,28	98.48 ± 4,37	212.33 ± 6.35	91.49 ± 5.47

Conclusions

- The presence of MSN or APTES-MSN improves the mechanical and barrier properties of BVPC films.
- The addition of the crosslinking enzyme (TG) to nanoparticle-containing BVPC films is able to counteract the improvement of mechanical properties and further increases barrier properties to both gas and WV.

Acknowledgement

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f. Contribution: Oral presentation
Congress: II QuimBioQuim, Universidad Rey Juan Carlos de Madrid (Spain) Date: 4/10/2017 - 5/10/2017

MEJORA DE LAS PROPIEDADES FISICOQUÍMICAS DE BIOFILMES PROTEICOS CON SILICA MESOPOROSA Y LA ENZIMA TRANSGLUTAMINASA

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Palabras clave: Biotecnología, Plásticos biodegradables, Sílica mesoporosa, Enzima Transglutaminasa

Abstract

El factor más limitante a la hora del uso de materiales plásticos biodegradables son sus limitadas propiedades fisicoquímicas para su uso industrial. La estrategia más extendida para mejorar dichas propiedades es la preparación de un Composite, un material que consiste en una matriz polimérica (fase continua) y un filamento o fase discontinua. El avance de la Biotecnología permite crear nuevos Composites con filamentos de dimensión menor de 100 nm (Nanocomposite). Estos demuestran mejores propiedades barrera y mecánicas, mejorando también la resistencia al calor comparado con los Composites convencionales.

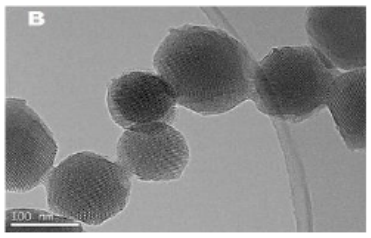

Fig. 1. SM

Fig. 2. Biofilme

En nuestro trabajo indagamos en una nueva estrategia para sintetizar nuevos materiales reforzados usando un concentrado proteico como matriz la sílica mesoporosa (SM) funcionalizada con (3-aminopropyl) trietoxisilano (APTES) como filamento. Con el fin de mejorar la estructura, la solución es proteica es incubada con la enzima transglutaminasa (TGase), que tiene la capacidad de "crosslinkcar" proteínas. Los resultados obtenidos demuestran que los filmes con las nanopartículas, tanto de SM como las funcionalizadas con APTES mejoran las propiedades mecánicas y barrera para ambos gases y para el agua. La incubación con la enzima reduce la permeabilidad.

Agradecimientos

Este trabajo ha sido financiado por el ministerio italiano de asuntos exteriores y la cooperación internacional "Fourth Executive Program of Scientific and Technological Cooperation" entre Italia y México, 2016.

3. Publications

a. Dalton Transactions. 2013, *42*, 14309-14314.

Dalton Transactions

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Seed-mediated growth of jack-shaped gold nanoparticles from cyclodextrin-coated gold nanospheres†

Cite this: *Dalton Trans.*, 2013, **42**, 14309

Alfredo Sánchez,[a] Paula Díez,[a] Reynaldo Villalonga,*[a,b] Paloma Martínez-Ruiz,[c] Marcos Eguílaz,[a] Iñigo Fernández[a] and José M. Pingarrón*[a,b]

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www.rsc.org/dalton

Branched gold nanoparticles were prepared by a seed-mediated approach using per-6-thio-6-deoxy-β-cyclodextrin capped gold nanospheres as seeds and a growth medium similar to those commonly employed to prepare gold nanorods, containing $AgNO_3$, ascorbic acid and cetyltrimethylammonium bromide. Novel jack-shaped gold nanoparticles (102–105 nm) were obtained at a specific range of Ag^+ ion concentrations (62–102 μM). The crystalline structure of these nanoparticles was confirmed by high-resolution transmission electron microscopy. The influence of the perthiolated β-cyclodextrin on the successful preparation of gold nanojacks was demonstrated. The jack-shaped gold nanoparticles showed strong absorption in the near infrared region and excellent catalytic activity for the electrochemical oxidation of H_2O_2.

Introduction

The synthesis of metal nanoparticles with defined morphology has attracted broad research interest and has undergone rapid development due to the unique electronic, optical, chemical and electrochemical properties of these nanomaterials.[1] In particular, a great deal of effort has been devoted to the development of methods for preparing branched and star-shaped gold nanoparticles.[2–7] These anisotropic nanomaterials show strong absorption in the near-infrared region, allowing their biomedical application as efficient contrast agents for biological tissues and cell imaging. Branched and star-shaped gold nanoparticles also show enhanced electromagnetic field intensity at their tip apexes, yielding higher signals in surface-enhanced Raman spectroscopy and thus favoring ultrasensitive detection.

Nanostars can be synthesized through a variety of seedless[5–7] and seed-mediated wet approaches[2–4] in aqueous and organic media, using diverse gold reducing conditions. In general, seed-mediated methods are preferred due to the possibility of using a great variety of nanoparticle seeds with different morphologies as well as the higher size- and shape-control of the nanostar growth.[8–12] As a general rule, the seed-mediated synthesis of branched gold nanostructures requires the use of selective capping agents during the colloid growth, due to the symmetric face-centered cubic lattice of gold nanoparticles.[8] In this regard, surfactants, DNA and polymers have been extensively employed as molecular templates for the sterically induced shape control of gold nanostar synthesis through seed-mediated strategies.[2–4,8–12] In addition, the type of reducing agent and the presence or absence of silver ions are also key factors in the rational syntheses of branched gold nanostructures.[1]

The surfactant cetyltrimethylammonium bromide (CTAB) combined with silver ions have been the capping agents more commonly employed to prepare anisotropic gold nanostructures. Nehl *et al.* used this capping mixture to prepare star-shaped gold nanoparticles of about 100 nm in high yield.[8] Vigderman and Zubarev described the synthesis of star fruit-shaped gold nanorods by using pentahedrally twinned gold nanorods as seeds.[13] Highly branched gold nanoparticles with controlled morphology were prepared by Sau and Murphy by using specific concentrations of CTAB, $AgNO_3$ and ascorbic acid as a reductant.[9]

Cyclodextrins are macrocyclic compounds with shallow truncated cone shapes usually employed as templates for the synthesis of polymeric and inorganic materials.[14] Cyclodextrins,[15] as well as their mono- and perthiolated derivatives,[16–18] have been also employed as ligands for the synthesis of water-

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c3dt51368h

b. Electrochemistry Communications 2014, *40*, 13-16

Electrochemistry Communications 40 (2014) 13–16

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Electrochemistry Communications

journal homepage: www.elsevier.com/locate/elecom

Short communication

Gold surface patterned with cyclodextrin-based molecular nanopores for electrochemical assay of transglutaminase activity

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ABSTRACT

A novel electrochemical method for measuring transglutaminase activity was reported. This approach was based on the patterning of gold electrodes with a mixed self-assembled monolayer of perthiolated β-cyclodextrin and 1-octanethiol. The proper functionalization of β-cyclodextrin with primary amino groups allowed it to act as amino-donor substrates for transglutaminase and molecular nanopores for the enzyme-controlled diffusion of $Fe(CN)_6^{3-/4-}$ to the electrode surface. Voltamperometric measurements allowed detection of transglutaminase in the range of 1.9–37 mU/mL with a sensitivity of 1.42 nA μL U^{-1} s^{-1}.

1. Introduction

Quantification of enzyme activity is relevant for clinical analysis, microbiological characterization, food quality assessment and the control of many industrial and biotechnological processes. Enzymes have been largely used in electroanalytical chemistry as catalytic bioreceptors and labels for the construction of a great variety of biosensors [1–4]. However, few attempts have been made to develop electrochemical methods to determine enzymatic activity [5–8]. Such approaches have been mainly based on the enzyme-catalyzed transformation or production of electroactive compounds, and have been mainly focused on redox enzymes [5]. For this reason, the establishment of electroanalytical methods to estimate the activity of non-redox enzymes deserves considerable attention.

In this context, transglutaminases (TGases, protein-glutamine γ-glutamyl transferases, EC 2.3.2.13) are particularly interesting enzymes. TGases are transaminases that catalyze the formation of ε-(γ-glutamyl)-lysine isopeptide cross-links into proteins via an acyl transfer reaction, where the γ-carboxyamide group of glutamine serves as the acyl donor and the ε-amino group of lysine serves as the acyl acceptor [9]. These enzymes are biologically important for the protection and prevention of body injury, tissue assembly and repair, and have also relevant pathophysiological role in several diseases [10]. TGases are also valuable biotechnological tools for food processing, neoglycoconjugates synthesis and edible films preparation [9,11–13]. In general, TGases are assayed by optical and radioactive methods [14], but to the best of our knowledge, electrochemical approaches have not been previously employed to quantify this enzyme.

In this communication we describe for the first time a voltammetric method to determine TGase activity. This approach is based on the patterning of a gold surface with molecular nanopores by coating with a mixed monolayer of perthiolated β-cyclodextrin (CD) and 1-octanethiol. Proper functionalization of the secondary face of β-CDs with primary amino groups allows them to act as amino-donor substrates for TGase as well as molecular nanopores for the enzyme-controlled diffusion of an electroactive probe to the electrode surface in the presence of a glutamine-donor substrate (Scheme 1).

2. Materials and methods

2.1. Reagents

TGase from *Streptoverticillium* sp. was purchased from Ajinomoto. *N*-Benzyloxycarbonyl-L-glutaminylglycine (CBZ), βCD and the other reagents were from Sigma.

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c. Chemical Communications 2014, *50*, 13356-13358.

ChemComm

COMMUNICATION

Nanochannel-based electrochemical assay for transglutaminase activity†

Iñigo Fernández,[a] Alfredo Sánchez,[a] Paula Díez,[a] Paloma Martínez-Ruiz,[b] Prospero Di Pierro,[c] Raffaele Porta,[c] Reynaldo Villalonga*[ad] and José M. Pingarrón*[ad]

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www.rsc.org/chemcomm

A novel electrochemical assay to quantify transglutaminase activity is reported. The assay is based on the enzyme-controlled diffusion of $Fe(CN)_6^{3-/4-}$ through amino-functionalized nanochannels of a mesoporous silica thin film on a Au surface in the presence of *N*-benzyloxycarbonyl-L-glutaminylglycine.

The establishment of rapid and reliable analytical strategies for enzyme activity quantification is a subject of broad interest due to the wide use of enzymes in clinical, industrial, basic research, chemical and environmental applications.[1] In this context, transglutaminases (TGases, protein-glutamine γ-glutamyl transferases, EC 2.3.2.13) are particularly interesting enzymes due to their ability to cross-link protein substrates. TGases are widely distributed in different organisms such as bacteria, plants, invertebrates and vertebrates.[2] In humans, TGases play important biological functions in the protection and prevention of body injury, tissue assembly and repair, and are also involved in the pathophysiological development of many diseases.[3] From an industrial point of view, TGases are relevant for food processing and for the preparation of edible films and neoglycoconjugates.[2,4]

TGases are commonly assayed through complicated optical and radioactive protocols[5] difficult to automate. Since electroanalytical methods are easily automated, miniaturized and integrated into portable and hand-held devices, they constitute ideal candidates to face the problem of enzyme activity quantification. In this context, we recently described a novel voltammetric strategy to quantify TGase activity by using cyclodextrin-patterned Au surfaces.[6] However, this approach implied a time consuming protocol for the synthesis of the oligosaccharide derivative and the sequential assembly of the sensing surface.

This communication describes for the first time a nanomaterial-based electrochemical assay for the quantification of TGase activity exhibiting remarkable advantages with respect to previous approaches. The rational of the strategy is illustrated in Fig. 1. It is based on the gold surface patterning with an array of well-ordered nanosized channels prepared by electrodeposition of a mesoporous silica thin film.[7] Subsequently, the outer film surface was functionalized with N^1-(3-trimethoxysilylpropyl)diethylenetriamine in order to provide the nanomaterial with amino-donor groups for TGase. The nanopores in the amino-enriched silica thin film acted as conductive channels for the diffusion of an electroactive probe to the electrode surface, thus providing a suitable electroanalytical signal. Then, the nanochannels were selectively gated by the catalytic action of TGase in the presence of the glutamine-donor substrate *N*-benzyloxycarbonyl-L-glutaminylglycine (CBZ). This produced a decrease in the accessibility of the electrochemical probe to the modified electrode surface and, accordingly, in the recorded voltammetric signal, by means of an enzyme-controlled diffusion mechanism.

The assembly of the electrode surface involved the former coating of the gold disk electrode (3.0 mm diameter) with a mesoporous silica thin film with vertically-aligned nanochannels by electrochemically-induced polycondensation of hydrolysed

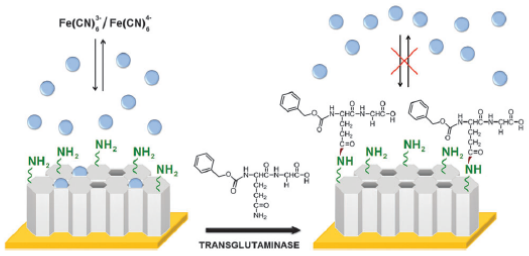

Fig. 1 Schematic display of the processes involved in the electroanalytical determination of TGase activity.

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Short communication

Mesoporous silica thin film mechanized with a DNAzyme-based molecular switch for electrochemical biosensing

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ABSTRACT

A novel electroanalytical strategy for copper and ascorbic acid detection was developed by using a nanostructured electrode surface mechanized with a DNAzyme-based molecular gate. This sensing interface was constructed by first electrodeposition of a mesoporous silica thin film on Au electrodes and further assembly of a Cu(II)-specific DNAzyme. The biosensing assay was based on the Cu(II) and ascorbic acid responsible activation of the DNAzyme, which acted as a molecular switch able to control the diffusion of the $Fe(CN)_6^{3-/4-}$ electrochemical probe through the nanochannels of the mesoporous film.

1. Introduction

The design of reliable and portable analytical devices for fast, accurate and *in situ* detection and monitoring of trace metals receives considerable attention due to the health and environmental impact of these compounds [1–3]. In this context, a special interest has been devoted to the development of sensor systems for copper. Several electrochemical, fluorescent, colorimetric and electrochemiluminescent sensors for copper have been designed [4–9]. Among these, excellent results have been reported for fluorescent biosensors based on specific Cu(II)-specific DNAzyme [10–12], but electroanalytical methods based on similar DNAzymes have not been described despite the high sensitivity, low cost, flexibility and miniaturization facility of electrochemical biosensors [13–17].

In this work we describe, for the first time, the assembly of an electrochemical biosensor for copper and ascorbic acid (AA) based on mesoporous silica thin films (MSTF) [18,19] mechanized with a Cu(II)-specific DNAzyme molecular switch. Although other electrochemical biosensing strategies based on DNA-blocked nanochannel arrays have been reported [20–22], the idea of using DNAzyme molecular machines on MSTF for heavy metals and antioxidant compounds determination has not been previously described.

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2. Materials and methods

2.1. Reagents and apparatus

All reagents and synthetic DNA oligonucleotides were from Sigma-Aldrich. The sequences of the nucleic acids were [23]: catalytic DNA end-group modified with a hexylamine-phosphoramidite moiety (5′-[AmC6F] TAA ATC TGG GCC TCT TTT TTA AGA AC-3′, DNA_1-NH_2 in Fig. 1), substrate DNA (5′-TTC TAA TDAC GATF TTA-3′, DNA_2 in Fig. 1).

Electrochemical measurements were performed using a FRA2 μAutolab Type III potentiostat/galvanostat. A three-electrode system was employed, by using a gold disk (2.0 mm diameter) modified with the functionalized MSTF, an Ag/AgCl/KCl (3 M) and a Pt wire as working, reference and counter electrodes, respectively. Atomic force microscopy (AFM) studies were performed with a SPM Nanoscope IIIa microscope. High resolution transmission electron microscopy (HRTEM) measurements were performed with a JEOL JEM-3000F microscope.

2.2. Preparation of electrode

A gold electrode was first coated with a MSTF [18,19]. To assemble the DNAzyme molecular switch, the MSTF-modified electrode was dipped into an ethanolic solution containing 2.26 mmol of (3-glycidyloxypropyl) trimethoxysilane for 1 h. The electrode was then washed with double distilled water and further dipped into a 1.0 μM solution of the catalytic DNA in the working buffer (50 mM HEPES buffer, pH 7.0, 0.5 M KCl, 0.5 M NaCl). After 1 h incubation at 20 °C,

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RESEARCH PAPER

Label-free electrochemical genosensor based on mesoporous silica thin film

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Abstract A novel label-free electrochemical strategy for nucleic acid detection was developed by using gold electrodes coated with mesoporous silica thin films as sensing interface. The biosensing approach relies on the covalent attachment of a capture DNA probe on the surface of the silica nanopores and further hybridization with its complementary target oligonucleotide sequence, causing a diffusion hindering of an $Fe(CN)_6^{3-/4-}$ electrochemical probe through the nanochannels of the mesoporous film. This DNA-mesoporous silica thin film-modified electrodes allowed sensitive (91.7 A/M) and rapid (45 min) detection of low nanomolar levels of synthetic target DNA (25 fmol) and were successfully employed to quantify the endogenous content of *Escherichia coli* 16S ribosomal RNA (rRNA) directly in raw bacterial lysate samples without isolation or purification steps. Moreover, the 1-month stability demonstrated by these biosensing devices enables their advanced preparation and storage, as desired for practical real-life applications.

Keywords Genosensor · Mesoporous silica film · Cyclic voltammetry · *E. coli* · 16S rRNA · DNA

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Introduction

Genosensor technology has demonstrated to be a powerful tool for clinical diagnosis, biomedical research, food quality assurance, and environmental monitoring [1, 2]. For this reason, the design of original, simple, and cost-effective analytical methods for the sensitive and accurate detection and quantification of specific nucleic acid sequences through hybridization mechanisms constitutes a main research topic in bioanalytical chemistry.

During last decades, a great variety of genosensors have been developed by using electrochemical, optical, calorimetric, and piezoelectric transduction strategies [3–6]. Although optical systems have been the most widely employed, electrochemical methods have great prospective due to the simplicity, easy preparation, relative low cost, and possibility to be miniaturized in portable point-of-care devices [7].

The assembly of original genosensors has been largely benefited by the use of novel nanomaterials and nanohybrids as advanced transducer elements. In this sense, a large number of electrochemical genosensors with improved analytical properties has been developed by using carbon nanotubes [8], graphene [9], metal and metal oxide nanoparticles [10–12], and other nanomaterials as constituents of the sensing interface. In general, these electrochemical genosensors involve the use of organic and metalorganic redox indicators [13, 14], enzymes [15, 16], or nanoparticles [17] as labeling

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